A comparison of the immune response and pathogenesis in sheep and cattle to *Toxoplasma gondii* infection

Irma Esteban-Redondo

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University of Edinburgh

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To Dr. Emiliano Esteban Velazquez

Abstract

Toxoplasma gondii is an extremely successful parasite capable of infecting all warm blooded animals. However, there is a wide variation between different animal species in their vulnerability to infection, providing a fascinating opportunity for comparative studies on the host/parasite relationship.

The main purpose of this thesis was to compare primary oral *Toxoplasma gondii* infection in two animal species, sheep and cattle, which are thought to differ in their respective vulnerability to infection. The parameters examined were the immune responses, pathogenesis and persistence of the parasite within tissues.

The rationale behind this study was based on reports that cattle seem to be more resistant to the outcome of *Toxoplasma* infection. Congenital disease is rarely reported and cattle are thought to harbour fewer parasite tissue cysts which may not persist for the life time of the host. Whereas in sheep, a primary infection usually results in the host remaining chronically infected for life and may result in abortion if primary infection occurs during pregnancy. It was hoped that these studies may indicate the critical factors involved in controlling *T. gondii* infection and increase our understanding of the events leading to persistence of the parasite within tissue cysts in the muscles and central nervous system of infected food animals. *T. gondii* tissue cysts in animal meat are an important source of infection for people, and recrudescence of parasites within tissue cysts in the central nervous system of immunosuppressed people, is one of the most common causes of death in AIDS patients. Host immune responses, in particular T cells and IFNγ, are known to play an important role in determining the outcome of *Toxoplasma* infection. Much of the data on host immune responses is derived from the study of laboratory mice which are extremely vulnerable to infection. There is comparatively little data from other animal species.

An initial experiment was conducted to determine an appropriate dose of *T. gondii* oocysts which would consistently produce detectable amounts of parasite cysts in sheep tissues. Groups of sheep were orally infected with a titrated dose of oocysts ranging from 10^3 to 10^5 and their tissues were examined 6 weeks later at post mortem. An infective dose of 10^5 sporulated oocysts gave the best result with *T. gondii* parasites being detected consistently in samples of heart and brain tissues. Two further experiments were conducted to compare *T. gondii* infection and host immune response in groups of sheep and cattle which were orally infected with either 10^3 or 10^5 *T. gondii* oocysts. Clinical responses, humoral and cell mediated immune responses were examined along with parasitaemia and the presence of *T. gondii* in various tissues at post mortem. The most striking difference between the sheep and the cattle was that *T. gondii* was more frequently and consistently detected in ovine tissues, in particular within brain and heart tissues, whereas parasites were not detected in the cattle samples. Although there was a marked difference in parasite detection, negative results do not necessarily exclude the possibility of persistent infection due to the limitations of sample size. Infection of sheep and cattle with either a low (10^3) or high (10^5) dose of oocysts provoked no more than a mild clinical response. The sheep developed an earlier febrile response which persisted longer than in the cattle. *T. gondii* was more frequently detected in the blood of sheep and cattle given the high dose of oocysts (10^5). Both, cattle and sheep, seroconverted following oral infection with antibody titres appearing to correlate with the infective dose. The higher the infective dose the higher the antibody titre. Specific antibody was detected earlier in sheep given 10^5 oocysts compared with 10^4 or 10^3. In agreement with previous studies on the immune response of sheep to *T. gondii,* an increase in the ratio of $CD8^+$: $CD4^+$ T cells occurred in peripheral blood following *Toxoplasma* infection. In cattle there was an early, brief increase in the percentage of $CD4^+$ T cells. The cytokine IFNγ was more frequently detected in blood plasma samples from cattle compared to sheep in the 3 weeks following infection with either 10^3 or 10^5 oocysts. In addition when *in vivo* primed peripheral blood lymphocytes were stimulated *in vitro* with specific *T. gondii* antigen the cells from cattle showed stronger proliferative activity which was detected more frequently compared with the

sheep cells under the same conditions. There was more IFNγ present in supernatants from activated cattle cells compared to sheep cells.

It is difficult to draw any precise conclusion as to why cattle may be less vulnerable to *T. gondii* infection than sheep. In general the cattle appear to be better able to control the parasitaemia resulting in fewer *T. gondii* cysts being detected in their tissues compared with sheep. Interestingly the cytokine IFNγ was more frequently detected either from plasma or activated cell supernatants in the infected cattle compared to the sheep. This cytokine is known to play an important role in protection against *T. gondii* and has been implicated as a factor involved in the differentiation of tachyzoites to bradyzoites (tissue cyst stage). Recent data would suggest that production of IFNγ can occur via NK cells very early in infection and that this may have a profound effect on the ability of the host to withstand infection. Future studies should perhaps focus on local responses at the very early stages of infection as measurement of peripheral immune responses may not be sufficiently sensitive.

Declaration

I hereby declare that I composed this thesis entirely by myself and that it describes my own research

Irma Esteban-Redondo

Edinburgh

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Abbreviations

AIDS `	Acquired immunodeficiency syndrome
bp	base pair
BSA	bovine serum albumin
Ci	curie
CMI	cell mediated immunity
CNS	central nervous syndrome
Con A	Concanavalin A
CPE	cytopathic effect
cpm	counts per minute
CT	cholera toxin
DHFR	dihydrofolate reductase
DIG	digoxigenin
d-NTP	deoxy nucleotide triphosphate(s)
T	thymine
A	adenine
G	guanosine
C	cytosine
DT	dye-test
d-UTP	deoxy uracil triphosphate
EIA	enzyme immunoassay
ELISA	enzyme linked immunoabsorbent assay
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
FIV	feline immunodeficiency virus
FITC	fluorescein iso thyocianate conjugated
G	gaugc
g	relative centrifugal force

GM-CSF	granulocyte-macrophage colony stimulator factor
GRA4	dense granule antigen
^{3}H	tritium
HBSS	Hank's balanced salt solution
H&E	haematoxylin and eosin
HIV	human immunodeficiency virus
IFAT	Indirect fluorescent antibody test
IFNγ	Interferon gamma
Ig	Immunoglobulin
IEL	intraepithelial lymphocytes
IL	interleukin
IMDM	Iscove's Modified Dulbecco's medium
i.p.	intraperitoneal
ISCOMS	immunostimulating complexes
KB	kilobase pairs
kDa	kilodalton (s)
LAT	latex agglutination test
LSA	lymphocyte stimulation assay
MBq	Mega Bequerels
MHC	major histocompatibility complex
MoAb	monoclonal antibody
MRI	Moredun Research Institute
N. caninum	*Neospora caninum*
NBT	nitroblue tetrazolium
NK	natural killer
OD	optical density
OPD	ortho-phenylenediamine
PAP	peroxidase antiperoxidase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffer saline

PBST	phosphate buffered saline Tween 20
PCR	polymerase chain reaction
PEG_2	prostaglandin E_2
r	recombinant
RBC	red blood cell
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
SAG1	surface antigen 1 or p30 antigen
sc	subcutaneous
se(m)	standard error (of the mean)
SDS	sodium dodecyl suphate
SFV	Semliki Forest virus
SI	stimulation index
TCID	tissue culture infective dose
TE	*Toxoplasma* encephalitis
T. gondii	*Toxoplasma gondii*
Th	T helper
TGR1	*Toxoplasma* dense granule 1
TNFα	Tumour necrosis factor alpha
Ts	temperature-sensitive
UV	ultraviolet
W.H.O.	World Health Organization
v/v	volume per volume
w/v	weight per volume

Chapter 1

Introduction

1. Introduction

Since the first description of the protozoan parasite *Toxoplasma gondii* by Nicole and Manceaux in 1908, numerous studies have been carried out to better our understanding of this parasite and its relationship with numerous intermediate hosts.

The definitive host of *T. gondii* is only found in members of the felidae family, however, this coccidian has a wide range of intermediate hosts and it is capable of infecting all warm blooded animals. The sexual stage of the life cycle only occurs in the enteroepithelium of felids but the asexual stage (tachyzoite) is able to infect and multiply in numerous organs within the intermediate and final host (Frenkel, 1973).

The consequences of infection are very variable among different animal species. The most vulnerable species include Marsupials and New World monkeys, in which acute infection can prove fatal (Dubey *et al.*, 1988c; Cunningham *et al.*, 1992). Whereas species, such as cattle, in which infection readily occurs but no disease is provoked are comparatively more resistant (Dubey, 1986b). In species such as humans or sheep, infection is generally unapparent in the adult host, but primary infection during pregnancy can cause serious disease or death in the foetus (Aparicio Garrido, 1978; Buxton, 1990). *Toxoplasma* infection has serious consequences in immunocompromised individuals such as the immunologically immature foetus, acquired immunodeficiency syndrome (AIDS) patients or people undergoing organ transplant and are therefore in receipt of immunosuppressive drugs (Luft and Remington, 1992).

The reasons for such a variation in species vulnerability to this protozoan parasite are not well understood. Perhaps the more vulnerable species become overwhelmed by the infection before the immune system is able to effectively respond. While in species, such as sheep and humans, the parasite provokes an effective immune response which is able to control the parasitaemia and the parasite

differentiates into bradyzoites, which will persist in the tissues for the life time of the host. Therefore in this case the host/parasite relationship resembles a stalemate situation which remains for the life time of the host unless some form of immunosuppression occurs. More resistant species such as cattle are thought to be able to control the infection very efficiently resulting in no clinical disease in the adult or abortion and there is controversy as to whether *T. gondii* persists in tissues (Dubey, 1986b).

A review of the literature reveals the extensive work conducted in the study of immunity to *T. gondii* infection largely carried out using experimentally infected mice (reviewed by Wong and Remington, 1993 and Gazzinelli *et al.*, 1993). While these studies have provided comprehensive knowledge on the immune mechanisms involved in controlling infection, one should be careful when extrapolating data arising from studies in mice to the situation in other species due to the different degrees of susceptibility to this parasite (Innes, 1997).

The main objective of this thesis is to compare host immune responses, pathogenesis and the development of tissue cysts in sheep and the more resistant species cattle following a primary oral infection with *T. gondii* oocysts.

It is hoped that these studies may indicate the critical factors involved in controlling *T. gondii* infection and maintenance of the tissue cysts in the muscles and central nervous system of infected animals. *T. gondii* tissue cysts in animal meat are not only an important source of infection for people, but recrudescence of such tissue cysts in the central nervous system of immunosuppressed people with *Toxoplasma* encephalitis (TE) is one of the most common causes of death in AIDS patients.

Chapter 2

Literature review

2. Literature review

2.1 Toxoplasma gondii*: historical background*

Toxoplasma gondii is an intestinal coccidian of felids with a world-wide distribution, affecting most warm-blooded animals, including man. *T. gondii* was first isolated in a rodent, *Ctenodactylus gundi*, in 1908 by Nicolle and Manceaux (1908). Charles Nicolle from the Pasteur Institute of Tunis, explored infantile splenomegaly already described in India as kala-azar, and the Oriental sore locally known as Gafsa pimple. He succeeded in establishing cultures of *Leishmania* parasites, and then embarked on an experimental and epidemiological study of the disease. Suspecting that rodents played the same role in the oriental sore as dogs did in the kala-azar, he captured rodents (gondi) in the Djerid desert. In two gondis which died and showed splenomegaly upon post-mortem examination, Nicolle and Manceaux (1908) observed parasites both free and within mononucleate cells. Because of its similarity with *Leishmania spp*, it was known as *Leishmania gondii.* However, the following year the name *Toxoplasma* was suggested for a new genus, different from *Leishmania* as the new parasite did not have a centrosome, and from the *Piroplasma* as *Toxoplasma* replicated in the white cells (Nicolle and Manceaux, 1909). *Toxoplasma gondii* was born. The name derived from the Greek *toxon,* bow or arc, referring to the lunate shape of the extracellular tachyzoite.

Splendore (1908) described the parasite in a laboratory rabbit (*Oryctolagus cuniculus*) in São Paulo in the same year, calling it *Toxoplasma cuniculi* (Splendore, 1909). Although Nicolle and Manceaux's and Splendore's descriptions agreed about the kidney-shaped and asymmetric morphology of the parasite, they differed on two points. Splendore identified schizogony or "multiple endogenous division", considering that the parasite formed true cysts, while Nicole and Manceaux denied

formation of a real wall and mentioned only simple artifacts resulting from the destruction of the host cell.

2.1.1 Toxoplasmosis in humans

Darling (1908) described what could be the first case of *Toxoplasma* infection in man in Panama although it was not until 1923 that the first case of toxoplasmosis was recognised in humans when Jankü (cited by Dubey and Beattie, 1988) found the parasite in the retina of a hydrocephalic child. In 1937, Wolf and coworkers described congenital *Toxoplasma* infection in the human neonate confirming the parasite as an important human pathogen. In the following years, Pinkerton and Weinman (1940) described the first case of fatal toxoplasmosis in adult human patient and Sabin defined the four characteristic symptoms of congenital toxoplasmosis: retinochoroiditis, cerebral calcification, hydrocephalus and mental retardation (Sabin, 1941). In the early 1950's the ocular form of toxoplasmosis in humans was claimed to be a consequence of congenital infection (Hogan, 1951). This was confirmed by Feldman (1952).

2.1.2 Toxoplasmosis in domestic animals

The first case of toxoplasmosis in domestic animals was reported in 1910, when Mello described a fatal case in a 4-month-old dog. He named the parasite described as *Toxoplasma canis* (Mello, 1910). However, owing to the similar morphology of *T. gondii* and *Neospora caninum,* perhaps this was the first case of neosporosis, which is now recognised to be a major cause of disease in puppies and young dogs. In the 1950's *T. gondii* was recognised as a significant cause of abortion in sheep by Hartley and Marshall (1957) and subsequently also in goats by Munday and Mason (1979), and pigs by Cole and associates (cited by Dubey, 1990).

2.1.3 Transmission and life cycle

As *T. gondii* was found in the blood, Chatton and Blanc (1917) suspected that the parasite may be transmitted via an arthropod vector. This group in Tunisia, as well as Woke and coworkers in USA, investigated transmission by arthropods, without success (Chatton and Blanc, 1917; Woke *et al.*, 1953). Transmission by the ingestion of the undercooked meat was suggested by Weinman and Chandler in 1954. This hypothesis of transmission through the ingestion of infected meat was experimentally verified in children in a tuberculosis hospital in Paris in 1965 by Desmont and collaborators. They compared the acquisition rates of *T. gondii* infection in children with tuberculosis before and after admission to the sanitarium. they found that acquisition of *T. gondii* rose after adding either barely cooked or undercooked meat to their diet. Later, Kean and collaborators (1969) described toxoplasmosis infection in medical students at Cornell University (Ithaca, New York), who ate undercooked hamburgers. The idea of transmission through infected meat was supported by the studies of the resistance of tissue cysts containing *T. gondii* bradyzoites to proteolytic enzymes, which would be present in the gut of the intermediate host (Jacobs *et al.*, 1960b). However, congenital transmission and carnivorism did not explain the fact that *T. gondii* seroprevalence rates in vegetarians were similar to those in non vegetarians (Desmonts *et al.*, 1965).

Clearly there was another route of transmission. Hutchison, (1965) put forward the theory that *T. gondii* was transmitted with the egg of the nematode *Toxocara cati.* However, this theory was invalidated when *Toxoplasma* infectivity was found in faeces of worm-free cats fed *T. gondii* (Frenkel *et al.,* 1969; Sheffield and Melton, 1969). Finally, the complete life cycle of *T. gondii* was described in 1970 following the discovery of the sexual life cycle of the parasite in the small intestine of the cat. Several groups described this cycle around the same time and confirmed cats as the definitive host of *T. gondii* (Frenkel *et al.*, 1970; Dubey *et al.,* 1970a, b).

2.1.4 Host-parasite relationship

Studies of the host-parasite relationship were first reported in 1937 when Sabin and Olitsky demonstrated that mice and rabbits experimentally inoculated with parasites and immune serum did not develop the infection. Since then most of the studies on immune responses to *T. gondii* have taken place using murine infection models and they have demonstrated that cell-mediated immune mechanisms are important in protective immunity (Beaman *et al.*, 1992; Gazzinelli *et al.*, 1993; Subauste and Remington, 1993). Specific antibodies to *T. gondii* are useful for diagnostic purposes. This was originally demonstrated with the development of the methylene blue "dye test" for detection of *Toxoplasma* specific antibodies described by Sabin and Feldman in 1948.

2.2 Life cycle

Toxoplasma gondii is an obligate intracellular parasite included in the phylum Apicomplexa, class Sporozoa and subclass Coccidia (Levine *et al.*, 1980). Although *T. gondii* is the only species in the genus *Toxoplasma, Isospora, Eimeria, Neospora* and *Sarcocystis* are other cyst-forming coccidia members of the same order.

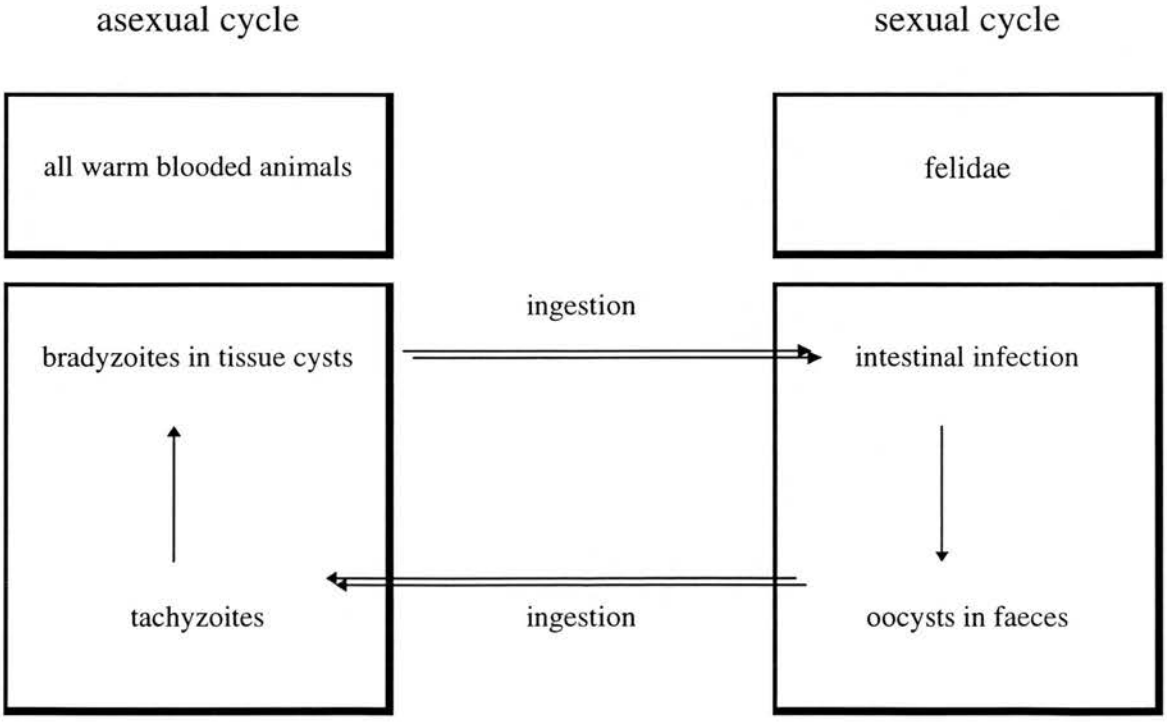
In brief, the life cycle comprises two stages; sexual cycle, (endodyogeny, endopolygeny, schizogony and gametogony) restricted to the epithelium of the small intestine of the cat (definitive host), and an asexual cycle (tachyzoites and bradyzoites) occurring in the intermediate hosts exhibiting little host or cellular specificity (Figure 2. 1).

2.2.1 Asexual cycle

Following the ingestion of contaminated food and water that contains any of the infectious stages of *T. gondii*: either tissue cysts (containing bradyzoites) or

oocysts (containing sporozoites), infection of the host occurs as a result of asexual multiplication of the parasite. The asexual cycle takes place in virtually all warm blooded animals including man, although there is considerable variation amongst different host species concerning the severity and consequences of the infection (Dubey and Beattie, 1988; Innes, 1997). Following ingestion, the outer walls of cysts or oocysts are disrupted by enzymatic degradation and the infective phases (bradyzoites or sporozoites) are liberated in the lumen of the intestine. They rapidly invade and multiply within surrounding cells, where they become tachyzoites. The parasite actively penetrates the host cells, becomes surrounded by a parasitophorus vacuole and multiplies by a process called endodyogeny (Joiner *at al.*, 1990). Multiplication continues until the host cell ruptures and the organisms are released to invade contiguous cells. Dissemination of the parasite occurs via the blood and lymphatic vessels. *T. gondii* can invade virtually all cells and tissues of the body (Krahenbuhl and Remington, 1982).

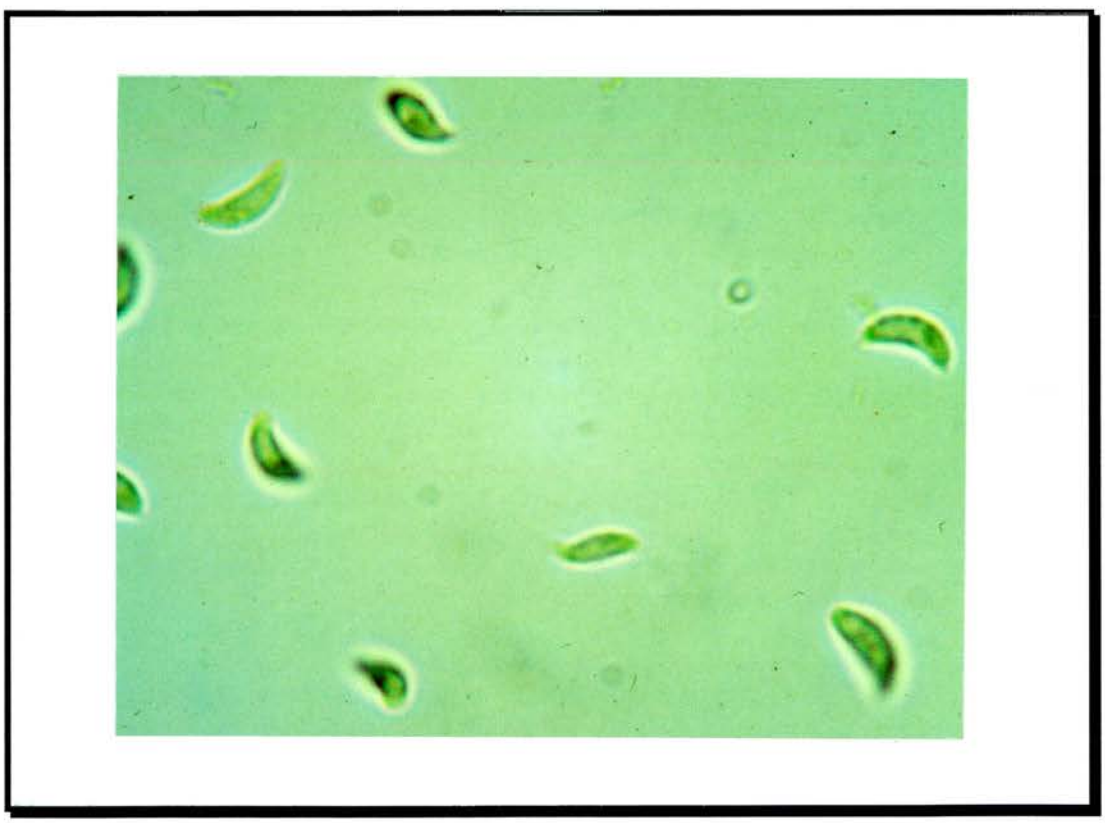
Figure 2. 1 The life cycle of the intracellular protozoan parasite *T. gondii*



2.2.1.1 Tachyzoites

The tachyzoite is the invasive rapidly dividing stage of the parasite responsible for the acute infection. It is crescent shaped with its anterior end pointed and its posterior end rounded and is approximately 7×3μm in size (Figure 2. 2). Although they are eukaryotic cells and contain Golgi apparatus, ribosomes and mitochondria, *T. gondii* require an intracellular habitat for survival and multiplication. They can infect phagocytic and non-phagocytic, nucleated and non nucleated cells (Werk, 1985).

Figure 2. 2 Crescent-shaped *T. gondii* tachyzoites in a cell free suspension



Photograph courtesy of Dr. D. Buxton

Tachyzoites are coated with three unit membranes forming the pellicle. The outer membrane is continuous, while the inner two membranes are closely opposed, discontinuous and end at the anterior and posterior ends in structures known as polar rings (Sheffield and Melton, 1968). The subpellicular cytoskeleton is composed of 22 microtubules that stretch from the anterior polar ring, running almost the length of the entire cell (Sheffield and Melton, 1968). The conoid, situated within the anterior end of the parasite, is composed of a truncated cone of spiral wound fibers. This structure is projected outwards during the parasite entry into host cells (Aikawa *et al.*, 1977). The four to eight rhoptries are club-shaped organelles that terminate in the conoid and have a secretory function (Schwartzman, 1986). The micronemes are tubular structures situated close to the rhoptries and are thought to have a secretory function. Current evidence would suggest that the conoid and rhoptries are involved in cell penetration (Chiappino *et al.*, 1984). Dense granules are organelles distributed throughout the cytoplasm and their contents are released into the protein-rich, reticulate network in the parasitophorous vacuole. Proteins from the dense granules are also released into the external environment defined as excretory/secretory antigens (Leriche and Dubremetz, 1990).

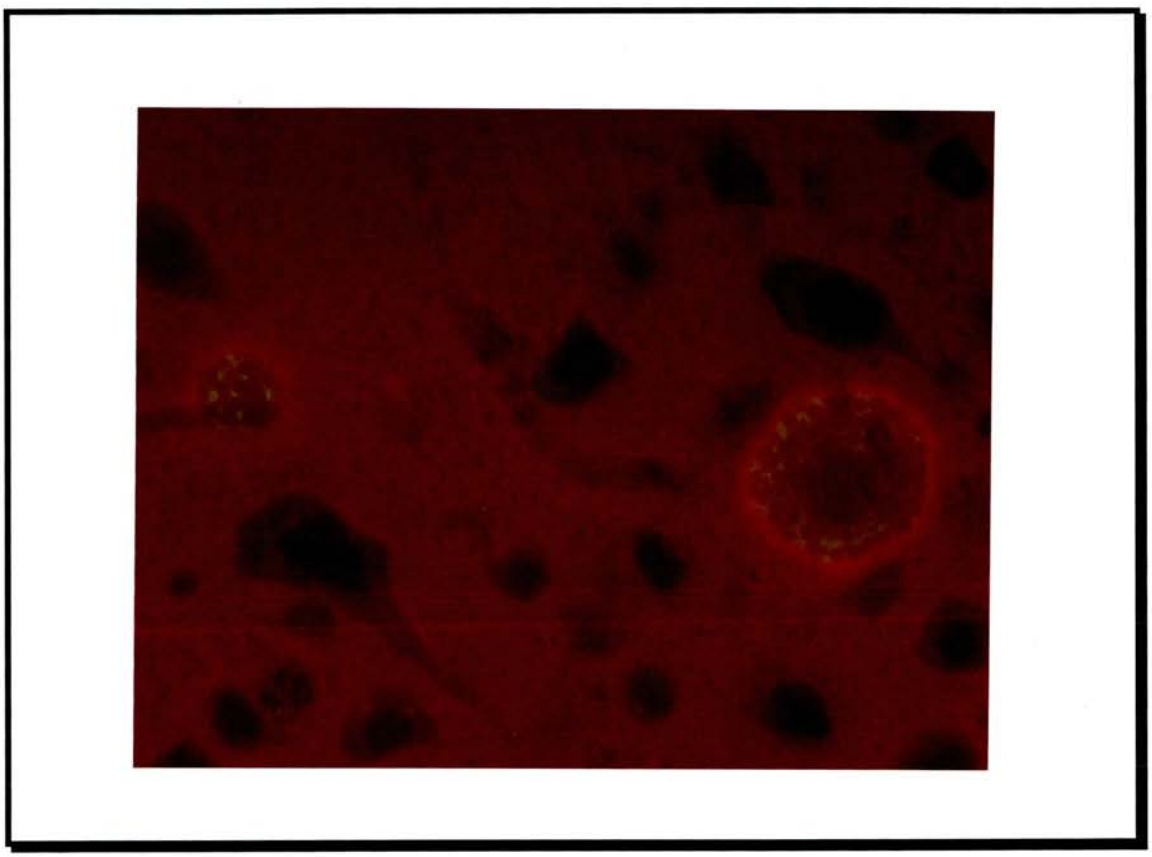
The parasite at this stage is contained inside the parasitophorous vacuole within the host cell and in this way is able to survive the normal hostile environment of the cell. This is because the parasitophorous vacuole neither fuses with lysosomes of the host nor does it become acidic (Joiner and Dubremetz, 1993).

2.2.1.2 Bradyzoites within the tissue cyst

Rapid multiplication of the parasite as tachyzoites continues until the host develops immunity to the parasite or in some cases the host dies. Thereafter a persistent infection is established, extracellular parasites are eliminated, intracellular multiplication slows down and the parasite accumulates in tissue cysts as bradyzoites. Bradyzoites are the slow multiplying forms of *T. gondii*, similar in size and shape to

the tachyzoites, and constitute the persistent stage of the parasite characteristic of the chronic (latent) infection (Figure 2. 3).

Figure 2. 3 Brain of a mouse showing bradyzoites within the tissue cysts. Parasites are labelled using an indirect immunofluorescence stain (——— = 50μm)



Photograph courtesy of Dr. D. Buxton

T. gondii parasites differentiate from the tachyzoite stage to the bradyzoite stage within the compartment created by the parasitophorous vacuole, which converts itself into a cyst. The bradyzoite is the stage that can initiate the sexual cycle in the gut of the cat (Dubey and Frenkel, 1976; Wong and Remington, 1993). Bradyzoites express stage-specific antigens which will allow discrimination between bradyzoites

and tachyzoites which are morphologically very similar (Kasper, 1989; Tomavo *et al.*, 1991). In the early stages of differentiation from tachyzoites to bradyzoites *T. gondii* parasites express both tachyzoite and bradyzoite specific antigens, indicating that this process does not occur simultaneously within a tachyzoite population (Soête *et al.*, 1993). Unlike tachyzoites, bradyzoites have a higher content of micronemes and amylopectin granules (Ferguson and Hutchison, 1987) and are relatively more resistant to pepsin and trypsin than tachyzoites, making them able to resist the digestive processes upon ingestion, despite the immediate disruption of the cyst by the enzymes (Jacobs and Melton, 1957; Jacobs *et al.*, 1960b).

The mechanisms involved in the transformation of the actively dividing tachyzoite to the slowly dividing stage, bradyzoite, are unknown. Using stage-specific monoclonal antibodies, the kinetics of conversion into bradyzoites has been analysed *in vitro* by several research groups (Jones *et al.*, 1986; Lindsay *et al.*, 1991; McHugh *et al.*, 1994). Spontaneous transformation from tachyzoites to bradyzoites has been observed in cell cultures, leading to the formation of tissue cysts. The conversion has been described as a gradual process, with a sequential expression of stage-specific molecules and gradual morphological conversion (Gross *et al.*, 1996). However, experiments conducted on differentiation *in vitro* have also shown that conversion from tachyzoite to bradyzoite stage can be induced by several immune factors (interferon gamma (IFNγ), nitric oxide) and metabolic, chemical or physical stress factors (alkaline pH, sodium arsenite, heat shock). In all of these studies it is important to note that *T. gondii* parasite stages are defined by the expression of stage specific antigens (Bohne *et al.*, 1993; Soête *et al.*, 1994).

2.2.1.3 Tissue cysts

Generally control of *T. gondii* within infected hosts is associated with cessation of tachyzoite replication and invasion, and then the formation of bradyzoites contained within the tissue cysts. Cysts, containing anything from a few tens to thousands of bradyzoites are 10 to 100 μm in size and are found most

frequently in brain, heart and skeletal muscle and in most cases persist for the life time of the host (Jacobs *et al.*, 1960a; Remington and Cavanaugh, 1965; Conley and Jenkins, 1981; Dubey and Thulliez, 1993). The outer membrane of the cyst is derived from components of both host cell and parasite. The intact tissue cyst does not evoke an inflammatory response. Immature tissue cysts may be detected as early as 1 to 2 weeks after challenge in experimental infections (Remington and Cavanaugh, 1965; Dubey and Frenkel, 1976; Conley and Jenkins, 1981; Dubey *et al.*, 1996).

The mechanisms involved in *T. gondii* stage differentiation and persistence of bradyzoites within tissue cysts are the subject of much research interest and are at present not well understood. Interestingly there have been reports in the literature that some species, such as cattle are able to eliminate or markedly reduce the numbers of parasite cysts in their tissues (Beverley *et al.*, 1977; Costa *et al.*, 1977; Fayer and Frenkel, 1979; Stalheim *et al.*, 1980b). Genetic investigations in mice have revealed that genes at the H-2L locus (class I genes) appear to regulate the number of cysts in the brains of mice orally infected with tissue cysts of *T. gondii* (Brown and McLeod, 1990).

Wong and Remington, (1993) reported evidence supporting the hypothesis that individual bradyzoites may escape from cysts without complete cyst wall disruption, proceeding to invade contiguous cells and thereby resulting in what is called a daughter cyst often seen in chronically infected mice. It is of major importance in understanding the host-parasite relationship to know whether tissue cysts are solely intracellular or can persist extracellularly (Wong and Remington, 1993) as well as why some species are able to reduce or even eliminate the number of tissue cyst from their tissues.

Because *Toxoplasma* encephalitis in AIDS patients is almost always the result of reactivation of a previous infection (Navia *et al.*, 1986; Luft and Remington, 1992), it was suggested that intermittent cyst rupture or leakage of parasite out of the cysts occurs most frequently in the central nervous system (CNS) and that the defective immune response results in uncontrolled proliferation and progressive encephalitis (Frenkel and Escajadillo, 1987). Development of TE in mice appears to

be controlled by a gene(s) located in the H-2D region, one of the class I genes of the major histocompatibility complex (MHC) (Suzuki *et al.*, 1991; Blackwell *et al.*, 1993).

The mechanisms that promote tachyzoite to bradyzoite interconversion in *T. gondii* are very complex and as yet poorly understood. However, new technologies and strategies will facilitate a better understanding of this important event in the pathogenesis of toxoplasmosis.

2.2.2 Sexual phase: enteroepithelial cycle

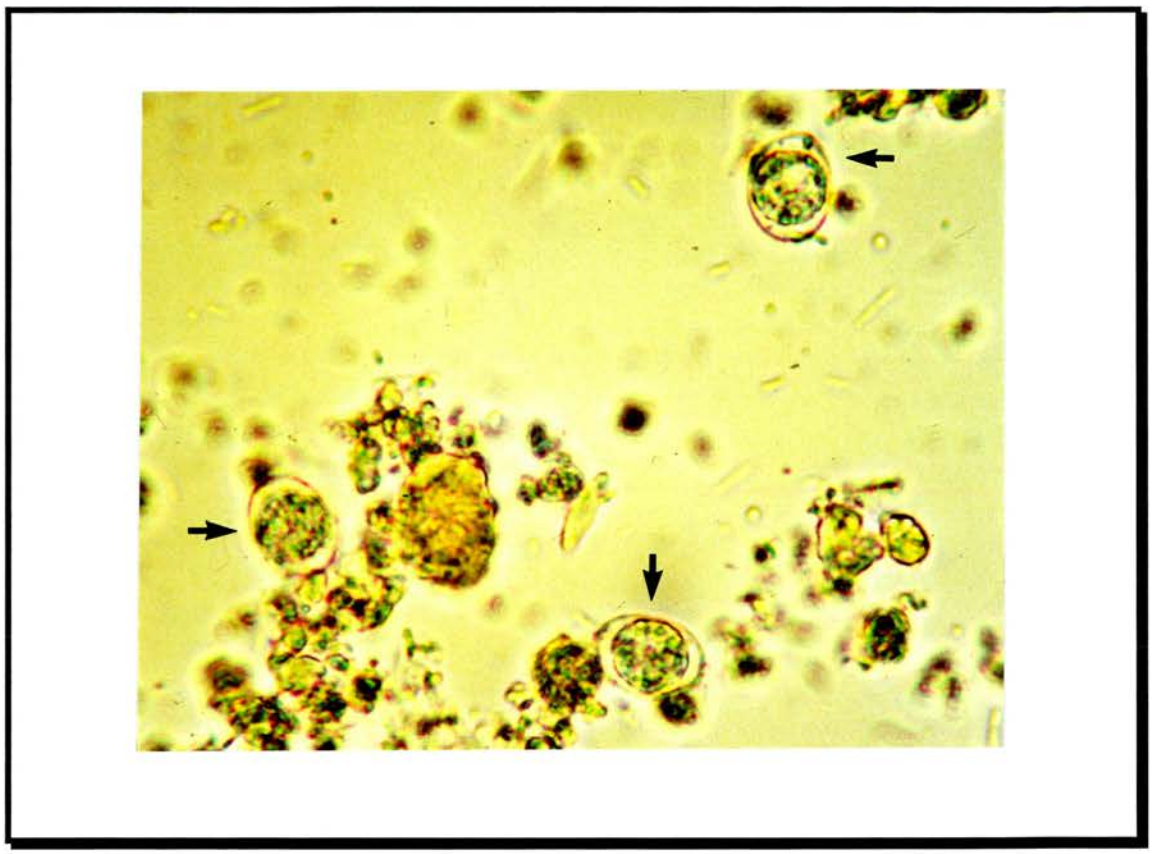
Initiation of the sexual cycle occurs mainly when nonimmune members of the Felidae family (domestic or wild) ingest tissues contaminated with the cyst form containing bradyzoites (Frenkel *et al.*, 1970; Dubey and Frenkel, 1972). Following ingestion, the outer walls of cysts are disrupted by enzymatic degradation and the infective phase (bradyzoites) are liberated into the intestinal lumen. The free bradyzoites enter the enteroepithelial cells and begin the differentiation into male and female gametes in the feline enterocyte. The female gamete (macrogamont) is subspherical and contains a single nucleus with several organelles. Mature male gamont (microgamont) is ovoid to ellipsoidal in shape. Microgametes are biflagellate organisms which consist mainly of nuclear material and contain a large mitochondrium. Male gametes are few and constitute 2 to 4 % of the mature gamont population. After the penetration of the macrogamont by the male gamete, a protective oocyst wall is formed around the zygote (termed oocysts).

2.2.2.1 Oocysts

When the oocysts are mature, they are shed into the intestinal lumen by the rupture of the intestinal epithelium. Unsporulated subspherical oocysts (10×12μm), contain the sporont, filling almost the whole oocyst (Dubey and Frenkel, 1972). Following excretion into the environment, they are non-infectious until sporulation

occurs (1-5 days depending on aeration and temperature) with three cellular divisions that results in eight infective sporozoites. Infectious oocyst (subspherical or ellipsoid, 11×13μm) contains two ellipsoid sporocysts (6×8μm), each of those containing four sporozoites ultrastructurally similar to the tachyzoites (2×6 to 8μm) (Dubey *et al.*, 1970a, b) (Figure 2. 4).

Figure 2. 4 *T. gondii* oocysts in a suspension of cat faeces



Photograph courtesy of Dr. D. Buxton

When ingested, oocysts are disrupted by the host digestive enzymes and the sporozoites are liberated into the intestinal lumen, commencing a new asexual cycle in the intermediate host. It has been reported that the time to shedding of oocysts in cats is 3-5 days after ingestion of tissue cysts and they are shed for 1-2 weeks and as

many as 10 million may be shed in a single day (Dubey *et al.*, 1970b; Dubey and Frenkel, 1972).

Oocysts are resistant to most ordinary environmental conditions and can live in the environment for months to years. The viability of oocysts is extended by higher humidity (Dubey *at al.*, 1970a, b; Frenkel and Dubey, 1972). In moist soil, infectivity has been shown to last for over a year (Frenkel *et al.*, 1975). Unsporulated oocysts are more sensitive to adverse temperatures than are sporulated oocysts. Exposure of unsporulated oocysts to 60-70°C for 10 seconds, 4°C for 90 days, -5°C for 14 days or -20°C for 1 day result in failure of the oocysts to sporulate, whilst sporulated oocysts retain their infectivity at -5°C for 120 days (Jackson and Hutchison, 1989).

2.3 Mechanisms of acquisition

2.3.1 Acquisition via tissue cysts

Ingestion of bradyzoites within the tissue cysts in meat from infected animals, alongside ingestion of oocysts from cat faeces contamination, are the main cause of postnatally acquired *T. gondii* infection in humans (Zuber and Jacquier, 1995).

Weinman and Chandler (1954) first suggested that undercooked meat from infected animals might be the source of *T. gondii* infection in people. The first definitive demonstration of *Toxoplasma* cysts in the tissues of sheep, swine and cattle took place in 1960 (Jacobs *et al.*, 1960a). They showed evidence that man can acquire the infection by consuming undercooked meat containing *T. gondii* bradyzoites within tissue cysts. The importance of this mode of transmission was demonstrated by a study of the seroconversion rates of children admitted to a tuberculosis hospital in Paris who were fed undercooked meat (Desmonts *et al.*, 1965). Cured pork has recently been shown to also be a source of *Toxoplasma* infection (Buffolano *et al.*, 1996).

Seroepidemiological studies provide evidence for the importance of undercooked meat from infected farm animals as a source of *T. gondii* infection as the highest seroprevalence of *T. gondii* among the human population is reported in countries like France, where undercooked meat is traditionally eaten (Zuber and Jacquier, 1995). Seroprevalence surveys of meat animals in many parts of the world indicate that the *T. gondii* infection rates vary considerably (Jackson and Hutchison, 1989). The actual presence of tissue cysts in the meat of food animals is the important factor in transmission. The parasite has been isolated only rarely from naturally infected bovine tissues although there have been occasional reports from Japan, Czechoslovakia, Italy and Argentina of successful isolation (Dubey, 1986b). Thus the actual role of beef in the epidemiology of *T. gondii* infection in the human population has been disputed. Whereas viable *T. gondii* tissue cysts have been isolated frequently from the tissues of naturally and experimentally infected sheep (Dubey, 1996), pigs (Dubey *et al.*, 1995), goats (Dubey, 1980) and wild game (Dreesen, 1990). It has been detected from soft tissues such as brain, skeletal muscle, small intestine, liver and diaphragm.

Tissue cysts have been found to persist in edible tissues of live animals for months (Dubey, 1986a). This chronic stage of the parasite is not resistant to conventional cooking at 67°C (temperature at the center of the piece of meat) (Garnham and Lainson, 1960). The ability of tissue cyst to survive for up to two months at 4°C (Jacobs *et al.*, 1960b) indicates that they are likely to survive the normal refrigeration process but they should be killed by commercial or domestic freezing procedures (Jacobs *et al.*, 1960b). Microwaving does not kill all *T. gondii* because of uneven cooking (Lundén and Uggla, 1992).

2.3.2 Acquisition via oocysts

Ingestion of oocysts is the only mode of transmission to herbivores, beside congenital infection, and one of the ways of infection in omnivores and carnivores (Hutchison, 1965; Hutchison *et al.*, 1969; Frenkel *et al.*, 1970; Hutchison *et al.*, 1970; Sheffield and Melton, 1970). The sole source of infection for sheep, cattle and

horses are oocysts. This stage of the parasite is also the major source of infection for pigs although it is possible for them to be infected by the ingestion of tachyzoites or bradyzoites present in meat (Dubey, 1986a).

The importance of ingestion of oocyst contaminated food and water as a route of infection in humans was confirmed by demonstration of the presence of antibodies to stage-specific oocyst/sporozoite antigens in the acute and convalescent sera of individuals infected in an outbreak associated with water contaminated with *T. gondii* oocysts (Wong and Remington, 1993).

Cats become infected as a result of ingesting infected tissues of intermediate hosts, such as small birds and rodents. A cat may shed millions of oocysts, and they are very hardy, capable of surviving in the soil for a year or more, increasing the likelihood of infection. They are resistant to most ordinary environmental conditions and can survive in cold climates but are less viable in arid environments (Dubey *et al.*, 1970b). Invertebrates like flies, cockroaches and earthworms can mechanically spread oocysts (Dubey *et al.*, 1970b).

Transplacental infection in cats rarely occurs and infection by ingesting oocysts is an unlikely source of infection in cats (Dubey and Frenkel, 1976). The prevalence of infection is highest in kittens and occurs when they begin to hunt small birds and small mammals such as rodents, particularly the latter since they can pass *T. gondii* infection from generation to generation without causing clinical disease (Beverley, 1959). Many cats will have seroconverted to *T. gondii* by adulthood (Wallace, 1971). It is unknown what percentage of previously infected cats repeat oocyst shedding on further exposure to *T. gondii*. Recent studies indicate that immunity against oocyst shedding is not life-long and oocyst shedding occurs in approximately 40% of the cats infected six years prior to a secondary challenge (Dubey, 1995). Oocyst shedding occurs in chronically infected cats immunosuppressed with extremely high doses of glucocorticoids (Dubey and Frenkel, 1974; Lappin, 1994). Whether other concomitant infections can induce re-shedding of *T. gondii* oocysts is not well known. It has been shown that coinfection

with feline immunodeficiency virus (FIV) does not affect the repeat shedding of oocysts (Lappin *et al.*, 1992).

Infection from direct contact with fresh faeces is very unlikely as oocysts have to sporulate to become infectious and fresh faeces cannot cause infection (Lappin, 1994). Domestic and barn cats in farm environments tend to nest and defecate in hay and straw mows and in grain stores thus providing the potential for direct infection of livestock feed with *T. gondii*. It has been demonstrated that sheep are frequently maintained in an environment significantly contaminated with oocysts. Certainly, fertilized fields with manure and bedding from building where cats live can be a source of infection (Blewett and Watson, 1983; Faull *et al.*, 1986).

2.3.3 Acquisition via tachyzoites

Tachyzoites are delicate organisms unable to survive outside the body of the host and are easily destroyed by gastric secretions (Jacobs *et al.*, 1960b). Although they are eukaryotic cells, they require an intracellular habitat for survival and multiplication. The parasites in this form are capable of infecting phagocytic and non-phagocytic, nucleated and non nucleated cells (Werk, 1985). The only ways in which tachyzoites can infect the host are by transplacental transmission from mother to foetus: inoculation of packed leukocytes during a blood transfusion or transplants or because of laboratory accidents (Dubey and Beattie, 1988).

During acute *Toxoplasma* infection, tachyzoites are distributed throughout the host tissues. Tachyzoites have been detected in several body fluids such as tears, nasal secretions, saliva, milk, vaginal secretions, semen, urine and faeces (Fayer, 1981) and isolated from the milk of cows, goats, sheep, pigs, dogs, cats, rabbits, guinea pigs and mice either naturally or experimentally infected with *T. gondii* (Jones, 1973). Although there is little danger of infection from milk because it is generally pasteurized or even boiled, unpasteurized goat milk has been reported as a source of human infection (Fayer, 1981).

Venereal transmission of *T. gondii* infection through infected rams is unlikely to be significant in the spread of the infection. The recovery of tachyzoites of *T. gondii* from the semen of experimentally infected rams was reported (Spence *et al.*, 1978), although the production of *T. gondii* infected semen appears to be restricted to a brief period shortly after infection (Teale *et al.*, 1982). Epidemiological evidence suggests that sexual transmission does not play a role in human toxoplasmosis, although organisms have been isolated from semen of seropositive men (Fayer, 1981).

2.3.3.1 Congenital infection

A case of congenital infection was first recorded in man in 1937 (Wolf *et al.*, 1939). It was not until early 1950's when transpacental transmission of *T. gondii* was described in sheep for the first time with the discovery of *Toxoplasma*-like organisms in placentas and foetuseses of several abortions (Hartley *et al.*, 1954).

Transplacental infection to the foetus occurs when mothers acquire *Toxoplasma* infection for the first time during pregnancy. However, in some hosts such as women, ewes, sows and nanny goats immunity following primary infection will protect the host sufficiently to prevent foetal infection or abortion occurring in subsequent pregnancies (Dubey and Beattie, 1988; McColgan *et al.*, 1988; Frenkel, 1990). However, repeated congenital infection occurs for ten or more generations in mice, rats, guinea pigs, and hamsters (Beverley, 1959; Dubey and Beattie, 1988). Thus, by passing *T. gondii* from generation to generation through congenital infection, rodents can provide a reservoir of infection in an area for a long time with the potential for infection of cats and the triggering of massive oocyst contamination of the environment (Buxton, 1990).

Infection with *T. gondii* is a major cause of abortion in important sheep-rearing areas such as New Zealand and the British Isles (Buxton, 1993b). Following initial multiplication of tachyzoites in the mesenteric lymph nodes (Dubey, 1984), there is a parasitaemia disseminating the infection to many tissues (Dubey and

Sharma, 1980; Reid *et al.*, 1982). In the pregnant ewe a progressive infection may establish in the gravid uterus. As a result of this parasitaemia tachyzoites are able to reach and parasitize the caruncular septa, the maternal tissue of the placentome. They then invade adjacent trophoblast cells of the foetal villi and from there, the rest of the foetus (Buxton and Finlayson, 1986). This is similar to human infection, in which the consequences of contracting *T. gondii* infection early in pregnancy may have the most severe consequences (Frenkel, 1990).

Although sporadic reports from several countries of neonatal deaths in cattle have been attributed to congenital *T. gondii* infection because of finding *Toxoplasma*-like structures in fetal bovine tissues, it is doubtful if *T. gondii* is a primary cause of bovine abortion (Munday, 1978; Stalheim *et al.*, 1980a, b; Dubey, 1984). Recent studies would suggest that perhaps these *Toxoplasma*-like structures reported in fetal tissues from cases of bovine abortion may be the closely related coccidian parasite *Neospora caninum. N. caninum* is now thought to be one of the major causes of infectious bovine abortion (Dubey and Lindsay, 1996).

2.4 Clinical toxoplasmosis and species vulnerability

Toxoplasma gondii is an obligate intracellular parasite, with an unusually wide range of intermediate hosts (Dubey and Beattie, 1988). The outcome of the infection is different depending upon the species (Table 2. 1). There is a wide spectrum of host/parasite relationships ranging from unapparent infection to fatal disease with various intermediate cases (Dubey and Beattie, 1988; Innes, 1997).

The evolution of Marsupials, such as kangaroos (*Macropus fuliginosus*) and koalas (*Phascolarctos cinereus*) as well as New World Monkeys like marmosets (*Marikina geoffroyi*), nightowl monkeys (*Aotus zonalis*), and squirrel monkeys (*Saimiri sciureus*) have taken place largely separated from the presence of the definitive host of *T. gondii*, the cat (Araujo *et al.*, 1973; Dubey *et al.*, 1988c; Hartley

et al., 1990; Miller *et al.*, 1992; Cunningham *et al.*, 1992). When these species do come into contact with *T. gondii*, mainly in zoos, infection is often fatal. *Toxoplasma* infection has been reported to be fatal in penguins (Mason *et al.*, 1991), which would not be expected in their natural environment to come in contact with the cats, and therefore the oocysts.

In less susceptible species such as humans or sheep, infection is generally unapparent in the adult host, but primary infection during pregnancy can cause serious disease or death in the foetus (Aparicio Garrido, 1978; Buxton, 1990). *Toxoplasma* infection has serious consequences in immunocompromised individuals such as the immunologically immature foetus, AIDS patients or people undergoing organ transplant and are therefore in receipt of immunosuppressive drugs (Luft and Remington, 1992). Whereas species, such as cattle, in which infection readily occurs but no disease is provoked are comparatively more resistant (Dubey, 1986b).

2.4.1 Postnatally acquired infection in immunocompetent individuals

When *T. gondii* infection is acquired by any immunocompetent host (with the exception of the very vulnerable species mentioned previously), acute infection usually results in a mild febrile response, dyspnea and anorexia, detected within two weeks of infection (Remington, 1974; Aparicio Garrido, 1978; Dubey and Towle, 1986). The parasite elicits a strong and persistent cell-mediated immune response, resulting in a stalemate situation mutually beneficial to the parasite and the host (Gazzinelli *et al.*, 1993). The parasite encysts and remains in the tissues, it is thought, for the life time of the host (Dubey, 1977; Dubey and Towle, 1986), but the apparent exception to this is cattle. Several workers have indicated that tissue cysts do not persist in bovine tissue, and that they are quickly eliminated or markedly reduced in number following infection (Beverley *et al.*, 1977; Costa *et al.*, 1977; Fayer and Frenkel, 1979; Stalheim *et al.*, 1980b; Dubey, 1983).

There are several situations in which the lack of an effective immune response against the parasite can result in serious consequences.

Table 2. 1 Comparative species vulnerability to *T. gondii* infection in intermediate hosts

Species	Vulnerability	Consequence of *T. gondii* infection
Marsupials **New World Monkeys**	High	Severe/Fatal
Human **Sheep** **Old World Monkeys** **Goats** **Pigs**	Intermediate	Moderate
Cattle **Horse** **Deer**	Low	Indistinct

2.4.2 Reactivated toxoplasmosis in immunocompromised individuals

The most prevalent form of *T. gondii*-induced disease reported currently in people is recrudescence of a chronic *Toxoplasma* infection often encountered in individuals who are immuno suppressed due to chemotherapy to permit tissue transplantation, for cancer treatment or in human immunodeficiency virus (HIV) infected patients (Luft *et al.*, 1984; Luft and Remington, 1992). Reactivated toxoplasmosis has emerged as the most common opportunistic infection in AIDS patients. The numbers of people at risk are high because of the prevalence of seropositive individuals in the population (Chang, 1996).

During chronic infection, it is likely that bradyzoites are slowly liberated from cysts, without complete cyst wall disruption, into the surrounding host tissue. In the immunocompetent host, these released parasites will provoke an influx of inflammatory cells during the early stages of the liberation process and development of microglial nodule at the site, that will readily destroy the parasite. Thus, the original stalemate situation will be restored (Wong and Remington, 1993). However, a defective immune response, will permit bradyzoites freed from cysts to behave like the fast replicating tachyzoites resulting in rapid parasite proliferation and severe tissue damage (Gazzinelli *et al.*, 1993). Consequently, *Toxoplasma* encephalitis in AIDS patients has been described, almost always, as the result of reactivation of a previous infection (Frenkel and Escajadillo, 1987). Recently, several authors have postulated that brain lesions are the result of reactivation in the peripheral organs with subsequent haematogenous spread of the tachyzoites to the CNS (Wong and Remington, 1993).

In nearly all the AIDS patients, TE was characterised by bilateral severe and persistent headache responding poorly to analgesics, alongside confusion, lethargy, ataxia and finally coma.

2.4.3 Prenatally acquired toxoplasmosis

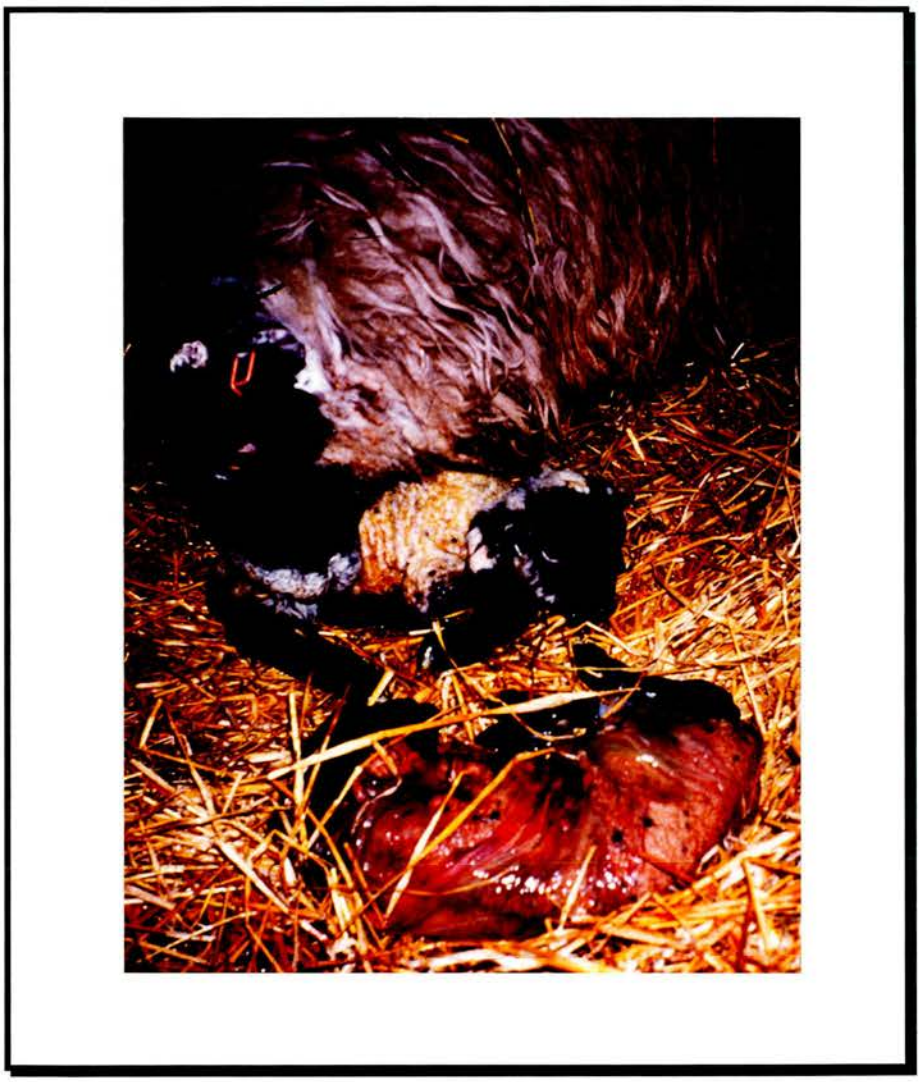
Another situation where the parasite can prove a dangerous opportunistic pathogen is to the developing foetus due to its immunological immaturity, as has been described in woman and sheep (Dubey and Beattie, 1988).

When acquired by a *T. gondii* naive individual during pregnancy, *T. gondii* can invade and cross the placenta to the immunologically immature foetus leading to severe tissue damage and in extreme cases abortion. As pregnancy advances the risk of clinical toxoplasmosis due to congenital *Toxoplasma* infection decreases as a consequence of the relative development of the fetal immune system (Buxton and Finlayson, 1986).

If the woman or ewe becomes infected in the first trimester of pregnancy, it is less likely that the parasite will be transmitted to the foetus but if transmission occurs the result is very severe, resulting even in death of the foetus. Infection later in pregnancy is more likely to result in fetal infection due to an increased blood supply to the foetus via the placenta, but the consequences of infection will be less severe due to the relative maturity of the foetal immune system. A triad of characteristic symptoms has been described in prenatal toxoplasmosis: hydrocephalus or microcephalus, intracraneal calcification and retinochoroiditis. This latter symptom is one of the most common clinical signs of human toxoplasmosis; usually results from a congenital infection, although it is generally not detected until much later in life (Aparicio Garrido, 1978; Frenkel, 1990).

Primary *T. gondii* infection in the ewe early in pregnancy is rapidly fatal to the foetus. Subsequent resorption of the foetus may be mistaken for infertility (Johnston, 1988; Buxton, 1990). It has been indicated that foetal death is due to the absence of an effective immune response able to control parasite multiplication (Buxton and Finlayson, 1986). Infection for the first time in late pregnancy generally has no clinical effect, the lamb will be born alive and immune although may be infected with *T. gondii* (Buxton and Finlayson, 1986; Buxton, 1990). A primary *T. gondii* infection of ewes in mid gestation can also result in stillborn, mummified, and /or weak lambs (Figure 2. 5).

Figure 2. 5 Ewe with stillborn lamb and weak live lamb following primary infection with *T. gondii* in mid pregnancy



Photograph courtesy of Dr. D. Buxton

Both in women and ewes, the mothers are not known to abort in subsequent pregnancies, either because of recrudescence of a chronic infection or as a result of a secondary infection. Therefore the host immunity induced by the parasite is sufficient

to protect against congenital toxoplasmosis in future pregnancies (Aparicio Garrido, 1978; McColgan *et al.*, 1988).

Unlike the situation in sheep, *Toxoplasma* infection does not appear to cause abortion or neonatal mortality in cattle under natural conditions (Dubey, 1986b) (Figure 2. 6). In 1953, what was thought to be clinical toxoplasmosis in cattle was reported for the first time in USA (Sanger *et al.*, 1953).

Figure 2. 6 Cow with healthy calf

Photograph courtesy of Dr. D. Buxton

The findings reported then have since been challenged as further examinations of the tissues failed to reveal *T. gondii* or related protozoan parasites

(Dubey, 1986b). Several studies of natural and experimental toxoplasmosis in cattle carried out since 1953 have shown that toxoplasmosis does not appear to cause abortion or neonatal mortality in cattle under natural conditions (Koestner and Cole, 1961; Dubey, 1986b), although in several cases the parasite has been isolated in mice inoculated with colostrum or unpasteurized milk (Dubey, 1986b). When pregnant cattle are inoculated with *T. gondii* oocysts or tissue cysts, transient fever and anorexia is detected and they give birth to healthy calves. The parasite was not isolated from the tissues of the newborn calves or placentas (Munday, 1978).

2.5 Pathogenesis of T. gondii *infection*

Since *T. gondii* was recognised in the early 1950's as a significant cause of abortion in sheep, extensive research has been carried out in sheep in order to understand the disease pathogenesis.

As it is beyond the scope of this thesis to review the pathogenesis of *T. gondii* in all hosts species, this section will be restricted to the pathogenesis of *T. gondii* infection and pathological findings in sheep and cattle.

2.5.1 Pathogenesis in non-pregnant animals

Immunocompetent non-pregnant sheep and cattle show only very mild clinical symptoms following *Toxoplasma* infection (Sanger *et al.*, 1953; Munday, 1978; Dubey and Towle, 1986; Dubey and Thulliez, 1993). *Toxoplasma gondii* is able to elicit a strong and persistent immune response which protects the host against rapid tachyzoite growth and consequent pathology (Gazzinelli *et al.*, 1993). As a consequence there are few lesions observed at post mortem examination in infected but otherwise healthy adults. Thus, much of the information on disease pathogenesis has come from experimental infections.

Following ingestion, sporulated oocysts are disrupted by the host digestive enzymes and the sporozoites are liberated into the small intestinal lumen of the host. Four days later the organisms (tachyzoites) will be found actively multiplying in the mesenteric lymph nodes causing marked enlargement, sometimes with focal necrosis (Dubey, 1984). They progress via the bloodstream and parasitize multiple tissues. In this early phase of the infection, the tachyzoites (the rapidly multiplying stage of the parasite) can be found in many different tissues (Dubey and Beattie, 1988). The parasite is found accompanied by mononuclear inflammatory reactions leading to small necrotic foci (Frenkel, 1988). The parasitaemia, which coincides with a febrile response of at least 41°C (Buxton *et al.*, 1988), has been detected from the fifth to the twelfth day following infection (Dubey and Sharma, 1980; Reid *et al.*, 1982; Wastling *et al.*, 1993).

In normal immunocompetent individuals, the parasite and the host immune system reach a compromise situation in which the tachyzoites are cleared from the hosts tissues and the necrotic foci regenerated, and no significant lesions are produced. The parasite differentiates to bradyzoites which are contained within cysts, where they are normally harmless to the host and serve to maintain the chronic infection. Because of their location within cysts, bradyzoites are less vulnerable to immune attack than tachyzoites. There is evidence that individual bradyzoites may escape from cysts without complete wall disruption, and in this way stimulate the immune response, which may be responsible for the long-lasting immunity induced by such chronic infections (Wong and Remington, 1993; Gazzinelli *et al.*, 1993). Unlike the situation in sheep, cattle are thought to harbour fewer *T. gondii* tissue cysts which do not necessarily persist for the life time of the host (Dubey, 1986b).

2.5.1.1 Pathological findings in **Toxoplasma** *infected sheep and cattle*

The lesions following experimental *Toxoplasma* infection of adult immnocompetent sheep and cattle occur most commonly in the nervous system, and lymph nodes. Where there is visceral involvement, pneumonitis, hydrothorax, ascites,

lymphadenitis, intestinal ulceration and necrotic foci in the myocardium, liver, spleen and kidneys may be observed (Radostits *et al.*, 1994).

2.5.1.1.1 Neuropathology of ovine and bovine *Toxoplasma* infection

Koestner and Cole (1961) observed that findings at post mortem examination in the ovine and bovine CNS, following *T. gondii* infection, were similar in both species, although the lesions in cattle are less severe. They reported a higher incidence of cerebral lesions in sheep than in cattle. The infection is characterised by focal necrosis and vascular damage in animals undergoing acute infection. Lesions comprise endothelial swelling, perivascular oedema, and proliferation of adventitia cell elements. Proliferating *Toxoplasma* organisms were present alongside numerous foci of necrosis, characterised by microglial proliferation. When chronic infection was studied, findings were identified as glial nodules, with mineralization of vascular walls. The lesions of the CNS were equally distributed and no preferred site was found.

2.5.1.1.2 Pathology of the lymph nodes

Buxton and colleagues (1981) outlined the findings in ovine popliteal lymph nodes after subcutaneous inoculation of sheep with tissue cysts of *T. gondii*. Severe pathological changes were described. Gross enlargement, loss of architecture, haemorrhages, some necrosis and the sinuses were full of plasma cells and numerous blast cells. In immune sheep, the changes provoked in lymph nodes by *T. gondii* were similar but less striking, with persistence of architecture and neither haemorrhages nor necrosis.

Essentially similar changes were described by Dubey (1984) in mesenteric lymph nodes after oral infection of sheep with oocysts. As early as four days post infection, the nodes were necrotic, infiltrated by neutrophils and there was lymphoid

depletion in the cortex. As the infection was progressing, histocytic infiltration was detected in the cortex and medulla.

2.5.2 Pathogenesis and clinical pathology of congenital toxoplasmosis in sheep

In naive, pregnant sheep, following ingestion of sporulated oocysts, the parasite will follow the same route as described in the previous section in a immunocompetent healthy adult. The parasite is disseminated via the blood stream to numerous tissues. In the pregnant ewe the infection will reach the gravid uterus. Tachyzoites can cross the placenta, parasitize the caruncular septa of the placentome, invading then the adjacent trophoblast cell of the foetal villi and from there the rest of the foetus, leading to severe tissue damage and in extreme cases death. On the uterine side, the maternal immunological response is suppressed, while the ability of the foetus and its placenta to recognize and respond to *T. gondii* develops progressively throughout gestation period (Buxton and Finlayson, 1986).

However, the outcome of infection will be influenced by the stage of gestation at which the infection takes place. *Toxoplasma gondii* infection in early gestation will have more severe consequences than infection late in pregnancy, when foetal immunity is relatively well developed (Blewett and Watson, 1983; Buxton and Finlayson, 1986; Johnston, 1988). *Toxoplasma* abortion in sheep usually occurs as a consequence of infection in early to mid pregnancy resulting in stillborn, mummified foetus, and/or weak lambs (Buxton, 1990).

2.5.2.1 Pathological findings in aborted material

2.5.2.1.1 Placenta

In general, the lesions described in the placenta vary depending on the time of the death of the foetus which will depend upon the transmission of infection from mother to foetus. Multiplication of tachyzoites in the cells of the caruncular septa of the placentome, causes focal inflammation and necrosis, allowing the parasite to

spread and invade the trophoblasts. When foetal death has taken place early in pregnancy, often no gross abnormality other than autolysis has been described (Hartley and Kater, 1963). The characteristic lesions consist of macroscopically visible multiple white spots present in the cotyledons. These correspond with foci of necrosis (2 to 3 mm in diameter). No lesion other than mild oedema was seen in the intercotyledonary area (Hartley and Kater, 1963; Beverley *et al.*, 1971b).

On histological examination, the earliest lesion seen consisted of edematous foetal villi with often the presence of a focal or diffuse mononuclear hypercellularity alongside hyperplasia and hypertrophy of the trophoblastic epithelium. Groups of *Toxoplasma* have been described in this area (Hartley and Kater, 1963; Buxton and Finlayson, 1986). Longer-standing lesions have been described as focal coagulative necrosis, desquamation of the hypertrophic trophoblast, necrosis of the contiguous maternal villi often with the presence of degenerating *Toxoplasma* (Hartley and Kater, 1963; Beverley *et al.*, 1971b; Buxton and Finlayson, 1986). In older lesion there are foci of caseous necrosis involving fetal and maternal villi, which often may be mineralized (Hartley and Kater, 1963). Tachyzoites may be seen at the edge of caseous necrotic lesions where they are often undergoing degeneration (Hartley and Kater, 1963).

2.5.2.1.2 Foetus

The pathological changes found in lambs born to *Toxoplasma* infected ewes vary depending upon the stage of gestation at which the dam acquires the infection. Hartley and Kater (1963) estimated that 35% of infected foetuses die before birth, 60% at the time of birth and 5% within a few hours of birth.

Infection in early gestation is rapidly fatal resulting in subsequent resorption of the foetus (Buxton and Finlayson, 1986; Johnston, 1988). However, a small mummified foetus often alongside still born and/or weak lambs will be found if the dam acquires the infection for the first time during mid gestation. Nonspecific gross lesions, such as anasarca and excessive fluids in cavities, will be found at

postmortem examination, possibly due to impaired nutrition and intrauterine death (Beverley *et al.*, 1971a, b).

Buxton and collaborators (1982) concluded, after examination of lambs born to ewes experimentally infected with *T. gondii*, that intrauterine infection of the foetus causes characteristic neuropathological changes in the foetus and that anoxia due to cotyledonary damage plays a significant role in the cause of death. The commonest pathological finding consists of perivascular cuffing by lymphoid cells and distinctive focal inflammation in the brain, a possible indication of a foetal immune response to the infection (Hartley and Kater, 1963; Buxton *et al.*, 1982). Extensive foci of leukoencephalomalacia mainly in the anterior cerebral periventricular white matter, and often associated with haemorrhages, are described in more than 50% of the examined cases. They consist of focal myelin loss, axonal swelling and degeneration. In older lesions, peripheral gliosis may be present, with central calcification (Hartley and Kater, 1963; Buxton *et al.*, 1982). In the foetal brain the parasite is seen in clumps attached to the vascular endothelium or associated with microglial nodules, as well as large non-encapsulated apparently extracellular clumps or cysts (Hartley and Kater, 1963).

Pathological changes have been reported in other organs. In the liver, there are focal granulomas, which may have central areas of necrosis, as well as large accumulations of lymphoreticular cells in portal triads and foci of extramedullary haemopoietic cells (Beverley *et al.*, 1971a; Buxton *et al.*, 1982). The adrenal gland shows infiltration of eosinophils which are often associated with macrophages (Hartley and Kater, 1963; Buxton *et al.*, 1982). Scattered small foci of mononuclear cells have been seen in lungs as well as pulmonic necrosis associated with *T. gondii* infection. In the heart interstitial myocarditis with infiltration by mononuclear cells between muscle fibers has been seen in some cases (Beverley *et al.*, 1971a).

2.6 Host-parasite relationship

In most cases infection with *T. gondii* induces a rapid and effective immune response which will protect the host but does not eliminate the parasite. A stalemate situation occurs where the host remains persistently infected with *T. gondii* bradyzoites within tissue cysts but generally the hosts suffers no ill effects from this situation. However, any change in the immune status of the persistently infected individual, e.g. any of the forms of immunosuppression discussed in 2.4.2, would result in reactivation of the parasite which would proceed to freely multiply until the host is overwhelmed and dies. Perhaps in species most vulnerable to *T. gondii* infection, such as marsupials and New world monkeys, the immune response is less efficient than in more resistant hosts such as sheep or cattle.

2.6.1 Host resistance to *T. gondii*

One important feature of *T. gondii* infection is the strong and persistent cell-mediated immune response elicited by the parasite, which protects the host against rapid tachyzoite growth and consequent pathology. Bradyzoites due to their protected location within tissue cysts, are therefore able to resist the immune response. Spontaneous release of bradyzoites from the protective environment of the cysts will stimulate the immune system maintaining a long-lasting immunity able to control the chronic *Toxoplasma* infection (Gazzinelli *et al.*, 1993). It is interesting to highlight that the strong immune response induced by the parasite is harmless to the host, and does not cause immunopathology in the immunocompetent host.

A review of host immune responses demonstrates the extensive research using murine models of *T. gondii* infection (Beaman *et al.*, 1992; Gazzinelli *et al.*, 1993; Subauste and Remington, 1993).

This section will focus on the host immune responses to *T. gondii* from studies using mice, sheep and cattle.

2.6.1.1 Immune responses in infected mice

Mice are very susceptible to *Toxoplasma* infection. Inoculation of mice with tissue samples is often used to help diagnose cases of toxoplasmosis. The mouse is a convenient and inexpensive animal model which has been extensively employed in studying various aspects of the immune response to *T. gondii* (McLeod *et al.*, 1993).

2.6.1.1.1 Mucosal immunity

The oral route is the natural site of entry of *T. gondii* (Frenkel *et al.*, 1969). The local tissues, underlying the intestinal epithelium are heavily populated with lymphoid cells from the mucosal immune system (Kraehenbuhl and Neutra, 1992) which are able to rapidly come into contact with the parasites during intestinal invasion (Chardès *et al.*, 1993).

Recent studies have been carried out to investigate mucosal immunity in the gut following *T. gondii* infection in mice. Evidence suggests that intraepithelial lymphocytes (IEL), (mainly CD8 α/β^+ Thy-1^+) play a major role at the mucosal surfaces as a first line of defence (Chardès *et al.*, 1994). Chardès and collaborators demonstrated the importance of IEL showing a decrease in the number of *T. gondii* tissue cysts in the brains of *Toxoplasma* infected mice when primed IEL cells were transferred to naive mice before infection. It has been reported that the effector mechanisms of this cell population is based on IFNγ production which limits the amount of iron available to the parasite and in this way inhibits *T. gondii* replication (Chardès *et al.*, 1994).

A local intestinal IgA response following *T. gondii* infection has been reported (Chardès *et al.*, 1990; Chardès and Bout, 1993). It has been established that a strong mucosal IgA response is induced against major surface *T. gondii* antigens (SAG-1, P30) following *T. gondii* oral infection of mice (Chardès *et al.*, 1990; Mineo

et al., 1993). IgA has been shown to inhibit the infection of host cells by *T. gondii* parasites (Mack and McLeod, 1992; Mineo *et al.*, 1993)

2.6.1.1.2 Innate resistance

Hauser and collaborators (1982, 1983) reported the ability of *T. gondii* to directly activate macrophages and natural killer (NK) cells. This activation occurs during the early stages of *Toxoplasma* infection before specific T cell induction occurs. NK cells have been identified as an important source of IFNγ and other cytokines produced by macrophages are involved in the initiation of the immune response (Sher *et al.*, 1993).

The production of IFNγ by NK cells has been attributed to the synergic activity of tumor necrosis factor alpha (TNFα) and interleukin 12 (IL-12 or NK stimulatory factor) produced by macrophages activated by *T. gondii* (Gazzinelli *et al.*, 1993).

Interestingly, it has recently been demonstrated that cell invasion is not the only method to activate macrophages, both live parasite and secretory/excretory antigens from *T. gondii* can induce the appropriate cytokine response resulting in IFNγ production by NK cells (Gazzinelli *et al.*, 1993).

The early activation of both macrophages and NK cells plays a very important role in establishing an early resistance against the rapidly dividing tachyzoites. The early production of IFNγ is also thought to provide the correct cytokine environment to induce the T helper1 (Th1) subset of $CD4^+$ T cells which are also thought to be important in immunity to *T. gondii* (Gazzinelli *et al.*, 1993)

2.6.1.1.3 Cell-mediated immunity (CMI)

Acquired resistance to the obligate intracellular parasite *T. gondii* has been demonstrated to be mainly dependent on CMI. Transfer of large quantities of serum

from chronically infected animals fails to protect naive mice against intraperitoneal (i.p.) challenge with virulent parasite strains (Pavia, 1986). Studies with IgM-suppressed mice, which lacked B lymphocytes and therefore failed to make antibodies, but had intact T cell functions, were able to control *Toxoplasma* infection (Frenkel and Taylor, 1982). Moreover, athymic nude mice which lack functional T cells were shown to be extremely susceptible to the parasite (Lindberg and Frenkel, 1977; Gazzinelli *et al.*, 1993).

Adoptive transfer studies using inbred strains of mice showed that both $CD4^+$ and $CD8^+$ T cells are relevant in the development of protective immunity (Suzuki and Remington, 1988; Gazzinelli *et al.*, 1991) and moreover that $CD8^+$ subset is the major effector population in controlling the infection (Suzuki and Remington, 1988; Parker *et al.*, 1991; Khan *et al.*, 1994).

2.6.1.1.3.1 T cells subsets involved in resistance to T. gondii

$CD4^+$ T lymphocytes are thought to be important in establishing immunity during early stages of infection (Araujo, 1991) or vaccination (Gazzinelli *et al.*, 1991) and it has been suggested that although $CD8^+$ T cells are the major effector T lymphocyte, their induction and optimal activity is dependent upon $CD4^+$ helper function (Chardès *et al.*, 1993; Denkers *et al.*, 1993) probably through the production of Th1 cytokines such as IL-2, IFNγ, TNF and granulocyte-macrophage colony stimulator factor (GM-CSF).

Two basic effector mechanisms by which $CD8^+$ T lymphocytes could limit *Toxoplasma* infection have been described. The first involves the production of IFNγ by the $CD8^+$ T cells, the activity of which is enhanced in the presence of IL-2 (Gazzinelli *et al.*, 1991; Denkers *et al.*, 1993). An adoptive transfer study showed that $CD8^+$ T cells were no longer protective if the recipient mice were treated with monoclonal antibodies to IFNγ (Suzuki and Remington, 1990). The second effector function involves direct lysis *T. gondii* infected host cells by MHC class I restricted $CD8^+$ T cells (Hakim *et al.*, 1991; Subauste *et al.*, 1991). The generation of these

effector cells seems to be dependent upon $CD4^+$ lymphocytes and IL-2 (Hakim *et al.*, 1991). It has been suggested that the releasing of soluble *Toxoplasma* antigen is important in maintaining $CD8^+$ lymphocytes in a primed state during long-term chronic infection via antigen presentation on bone marrow macrophages (Denkers *et al.*, 1992).

2.6.1.1.3.2 Interferon gamma

Interferon gamma plays a crucial role in host resistance to *T. gondii* (Subauste and Remington, 1991). If this cytokine is depleted from previously vaccinated mice they become vulnerable to *Toxoplasma* challenge (Gazzinelli *et al.*, 1991). Depletion of IFNγ in chronically infected mice using specific monoclonal antibodies has lead to the reactivation of previously dormant tissue cysts in the brain causing similar pathology to that described in AIDS patients with TE (Suzuki *et al.*, 1989). IFNγ may also be important in the effector function of activated $CD8^+$ cells by activation of other cell types, e.g. macrophages, to destroy tachyzoites released from the lysed target cells (Subauste and Remington, 1991). Interferon gamma is also known to be involved in the maturation of the $CD8^+$ cells into effector cytotoxic cells (Zanovello *et al.*, 1988).

2.6.1.1.4 Humoral immunity

Although cell mediated immunity is generally thought to be more important in recovery from a primary *T. gondii* infection, humoral immunity also has an important role. It is known that specific antibodies can destroy tachyzoites in the presence of complement (Sabin and Feldman, 1948; Schreiber and Feldman, 1980). Tachyzoites opsonized by antibody or complement are unable to prevent fusion of their parasitophorous vacuole with the host cell lysosomes and thus are unable to evade the normal killing mechanisms of macrophages (Joiner *et al.*, 1990). It has been also suggested that *Toxoplasma* specific antibodies may inhibit the invasion of

cells by blocking the activity of secretory-excretory substances responsible of promoting the host cell penetration (Schwartzman, 1986).

The passive administration of immune sera containing polyclonal antiserum confers little or no protection against *T. gondii* challenge to mice (Krahenbuhl *et al.*, 1972; Pavia, 1986). Moreover, transfer of monoclonal antibodies against the major surface protein of *T. gondii* P30 to naive recipients has conferred some protection against challenge infection (Johnson *et al.*, 1983; Sharma *et al.*, 1984). It has also been demonstrated that immune serum conferred partial immunity to B-cell-deficient mice (Frenkel and Taylor, 1982). It is important to mention that specific IgA responses in the gut mucosa are thought to be an important barrier to oral infection (McLeod and Mack, 1986).

In summary antibodies are important in resistance to *T. gondii*, in particular during a secondary infection, acting together with CMI.

2.6.1.2 Immune response in infected sheep

Congenital toxoplasmosis in the sheep is very similar to congenital human toxoplasmosis. Tansplacental transmission of the infection will only occur if the ewe acquires the infection for the first time during pregnancy, and the parasite is not transmitted to the foetus in chronically infected immunocompetent animals (McColgan *et al.*, 1988). Consequently the use of sheep to study *T. gondii* infection is not only of direct relevance to control the disease in this species but may be a more relevant model for studying human congenital *Toxoplasma* infection than relying entirely on mouse models where repeat vertical transmission is known to occur (Beverley, 1959).

Early reports suggested that infection with *T. gondii* induces an immune response in sheep (Olafson and Monlux, 1942; Wickman and Carne, 1950). In both experimental and natural infection with *Toxoplasma* specific seroconversion occurs (Hartley and Marshall, 1957; McColgan *et al.*, 1988). Antibody levels in sheep infected with a complete strain of *T. gondii* may remain steady for 5 months after

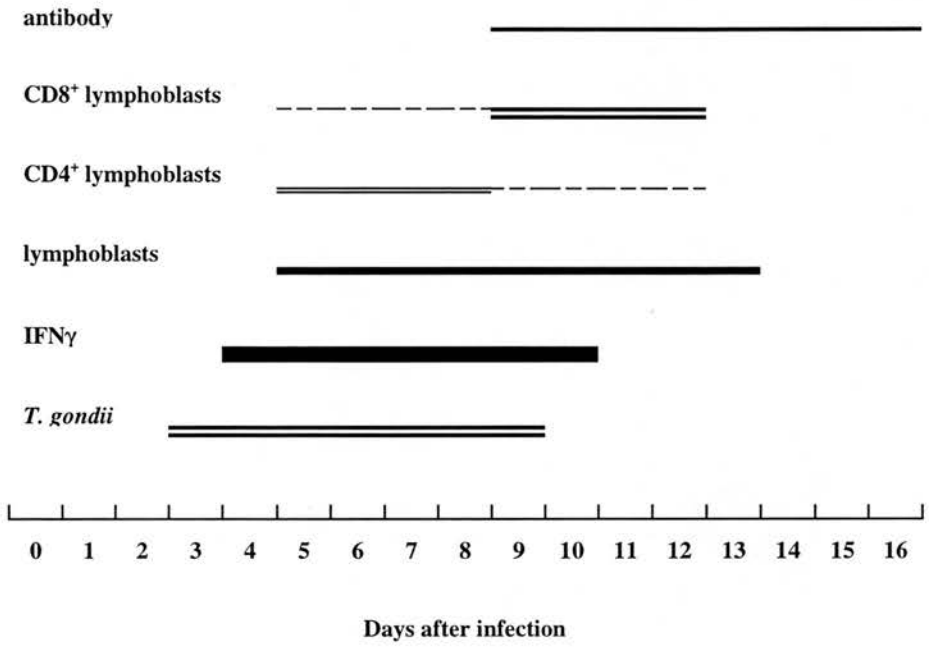
infection (McColgan *et al.*, 1988). In a primary infection *Toxoplasma* specific IgM antibodies are initially detected, followed by specific IgG antibody (Miller *et al.*, 1982; Blewett *et al.*, 1983).

Studies carried out at Moredun Research Institute (Edinburgh, UK), using experimentally infected sheep, have focused on the study of the immune response in sheep with the incomplete S48 strain of *T. gondii* (Wilkins *et al.*, 1987). The S48 *T. gondii* parasites lack the ability to differentiate to bradyzoites and hence cannot form tissue cysts (and therefore to establish persistent infection). Following subcutaneous inoculation of naive sheep with S48 tachyzoites, the *in vivo* immune response elicited by the parasite in a peripheral lymph node draining the site of challenge was monitored (Figure 2. 7).

The humoral response was dominated by antibody recognizing a 30-32 kDa band, probably corresponding to the major surface glycoprotein of *T. gondii* tachyzoites designated P30 or surface antigen 1 (SAG1) (McLeod *et al.*, 1991). The antibodies were detected from day 9 following a primary *T. gondii* infection. However, secondary challenge resulted in a immunological memory response with antibody being detected 3-4 days after infection compared with 7-8 days after primary infection (Wastling *et al.*, 1995).

Examination of cell output from the lymph node showed an initial decrease, presumably due to the phenomenon called cell shutdown which takes place in lymph nodes following antigenic challenge (Hall and Morris, 1965; Buxton *et al.*, 1994). Then the cell output from the lymph node increased and largely comprised lymphoblast cells responding to the parasite infection. Phenotypic analysis of lymphoblast cells in the efferent lymph showed that the cells responding to infection were mainly T cells. Initially those with a $CD4^+$ phenotype producing IFNγ ,were more numerous than $CD8^+$ T cells but at around day 9 to 10 after infection the latter became predominant. The disappearance of the parasite from the lymph also occurred at this time (Innes and Wastling, 1995).

Figure 2. 7 Host-parasite relationship detected in efferent lymph from a peripheral lymph node draining the site of subcutaneous challenge in a sheep undergoing primary infection with tachyzoites of the S48 strain of *T. gondii* [1]

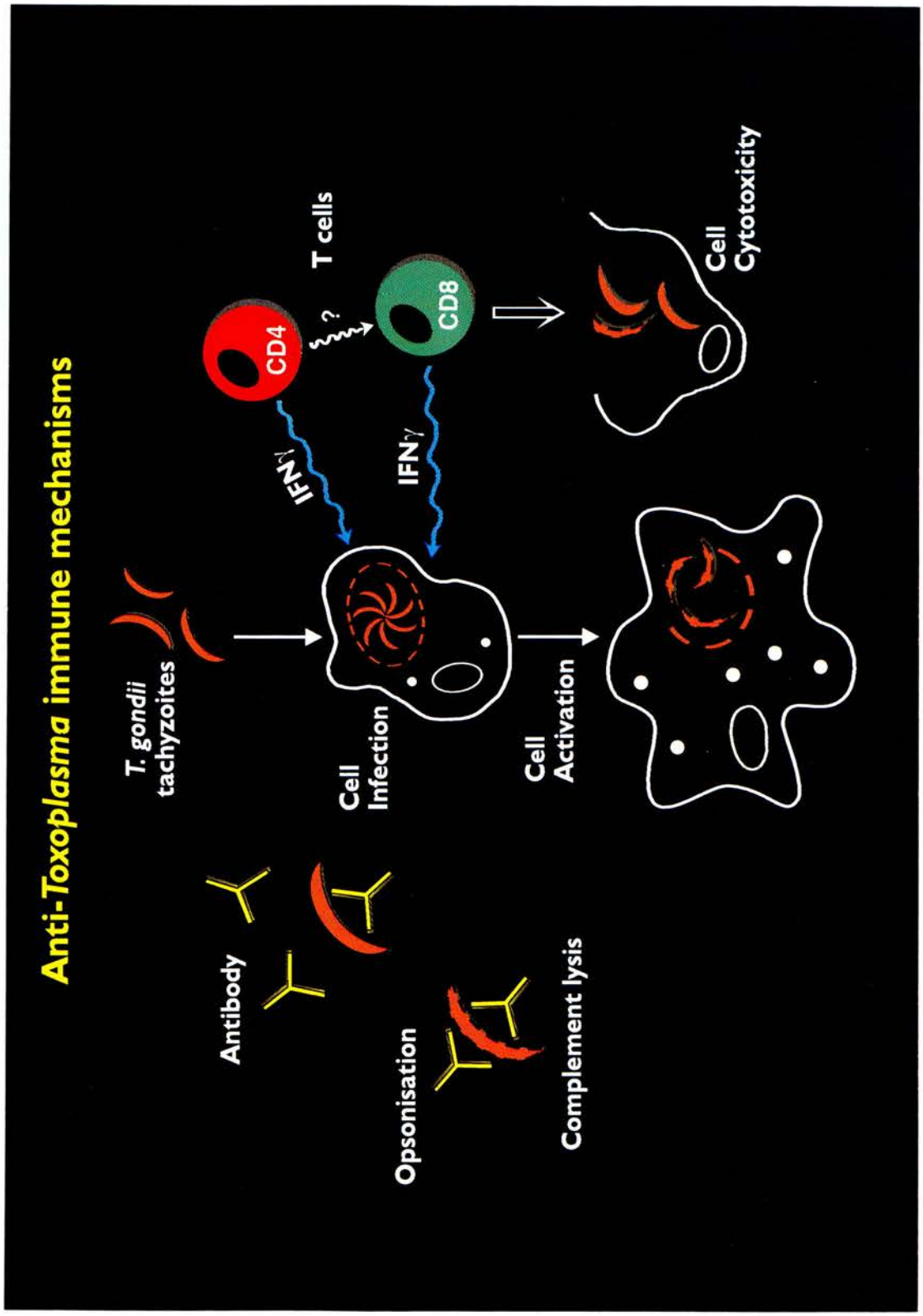


IFNγ was detected in cell-free lymph supernatant from days 3 to 10 after primary infection but appeared in lymph as early as 24 hours after secondary challenge (Innes *et al.*, 1995b). Both $CD4^+$ and $CD8^+$ T cell subsets have the potential to produce IFNγ after appropriate stimulation (Morris *et al.*, 1982; Mosmann and Coffman, 1987). It has been demonstrated that $CD8^+$ T cells from responding efferent lymph are able to inhibit *Toxoplasma* multiplication *in vitro*

[1] From Innes and Wastling, 1995.

(Innes *et al.*, 1995a). These observations suggest that a T cell response combined with IFNγ production plays a major role in immunity to *T. gondii* infection in sheep. It is generally thought that the $CD4^+$ T cell subset is required for optimal induction of the $CD8^+$ T cells (Suzuki and Remington, 1988; Gazzinelli *et al.*, 1991; Parker *et al.*, 1991; Innes *et al.*, 1995a). These *in vivo* observations are further supported by *in vitro* studies which have shown that recombinant ovine IFNγ will inhibit the growth of *T. gondii* in ovine cells (Oura *et al.*, 1993).

Figure 2. 8 Possible anti-*T. gondii* mechanisms of components of the host immune system (From Innes and Wastling, 1995)



2.6.1.3 Immune response in infected cattle

The reasons for the existing diversity in vulnerability to *Toxoplasma* infection among different species are not well understood, although it is likely that the host immune system plays an important role.

There is very little published data on immune responses in *T. gondii* infected cattle. The majority of information is on studies of seroprevalence and seroconversion in experimentally infected animals.

In cattle, seroprevalence rates are difficult to establish due to problems of specificity with the serological tests used (Dubey, 1986b). The Sabin-Feldman dye test (DT), thought to be the most reliable test for human sera was found to be unsuitable for use in cattle as it can give a false positive reaction due to a naturally occurring globulin, thought to be IgM. This globulin can be partially inactivated by heating the sera to 60°C for 60 minutes (Dubey *et al.*, 1985). Furthermore, in experimentally infected cattle DT titres increase briefly during the first month after inoculation before returning to baseline levels. There have been a number of reports indicating that the indirect fluorescent antibody test (IFAT) may be as sensitive as the dye-test (Munday and Corbould, 1971; Munday, 1978; Uggla, 1986).

Cattle experimentally infected with *T. gondii* develop specific antibodies within four weeks of infection (Beverley *et al.*, 1977; Costa *et al.*, 1977; Dubey *et al.*, 1995; Fayer and Frenkel., 1979; Munday, 1978). In cattle there is an age effect in antibody development with calves producing higher and longer lasting antibody titres than adult cattle, following experimental challenge with the same dose of *T. gondii* (Dubey *et al.*, 1985; Dubey, 1986b).

Under natural conditions it has been shown that sheep grazing on a pasture alongside cattle developed significant antibody titres to *T. gondii* as measured by IFAT while none of the calves became sero-positive (Munday and Corbould, 1979) corroborating once more the different susceptibility existing between these two species.

There have been no studies of CMI in *T. gondii* infected cattle, to my knowledge.

2.7 Diagnosis of T. gondii *infection*

Diagnosis of *Toxoplasma* infection can be made using a variety of methods including histopathology, serology, isolation of the parasite and amplification of specific deoxyribonucleic acid (DNA) by polymerase chain reaction (PCR).

2.7.1 Clinical signs

Diagnosis of *Toxoplasma* infection by clinical signs or macroscopic pathological changes is difficult since these are nonspecific and the symptoms are common to other infections.

2.7.1.1 Clinical signs of Toxoplasma *infection in man*

In the immunocompetent person *T. gondii* only rarely causes illness. Symptoms of infection include listlessness, fatigue, headache, muscle and joint pains, and slight fever. Because these symptoms are common to other diseases, toxoplasmosis is often unrecognized.

2.7.1.1.1 Postnatally acquired toxoplasmosis in human

2.7.1.1.1.1 Lymphoglandular toxoplasmosis

Lymphadenitis is the most frequently observed clinical sign in immunocompetent persons. The most frequently involved nodes are deep cervical ones. When infected the nodes are tender, discrete but not painful (Dubey, 1977).

2.7.1.1.1.2 Toxoplasma *encephalitis*

The most serious complications of toxoplasmosis have been described in patients whose immunity is suppressed, e.g. patients undergoing immunosuppressive therapy for tissue transplantation or cancer therapy, or in people who are immunocompromised as a result of HIV infection (Luft and Remington, 1992). Clinically, patients may have a severe headache, disorientation, drowsiness, hemiparesis, reflex changes, convulsions, and may become comatose, developing encephalitis and brain abscesses (Navia *et al.*, 1986).

2.7.1.1.2 Congenital toxoplasmosis

When a pregnant woman acquires the infection for the first time during pregnancy, she may transmit it transplacentally to her foetus, sometimes resulting in death of the foetus. Very often signs and symptoms are not apparent until weeks, months, or even years after birth.

While the mother rarely has symptoms of infection, she does have a temporary parasitaemia (Dubey and Beattie, 1988). Severely diseased children may have the full triad of signs proposed by Sabin *et al.* (1952): hydrocephalus or microcephalus, intracraneal calcification and retinochoroiditis. However, the most common consequence of congenital toxoplasmosis is ocular disease, affecting mainly

the posterior chamber and causing retinochoroiditis. The punctate lesions described on the retina are usually harmless and may not be detected until adulthood (Perkins, 1973).

2.7.1.2 Clinical signs of Toxoplasma *infection in sheep*

It has been generally reported that postnatal acquired *Toxoplasma* infection in adult immunocompetent sheep only provokes a mild clinical response, accompanied by temperature response and moderate diffuse symptoms, most often remaining unnoticed (Blewett *et al.*, 1982; Miller *et al.*, 1982; Dubey, 1984; McColgan *et al.*, 1988; Buxton *et al.*, 1991).

2.7.1.2.1 Congenital toxoplasmosis

When a pregnant ewe becomes infected for the first time during pregnancy, only a mild febrile response and dyspnoea may be detected. Much of the pathology is found in the foetus.

The outcome of the infection is influenced by the stage of gestation when it occurs (Blewett and Watson, 1983).

2.7.1.3 Clinical signs of Toxoplasma *infection in cattle*

A review of the relevant literature demonstrates that although cattle can readily become experimentally infected with *T. gondii* (Costa *et al.*, 1977; Munday, 1978; Dubey, 1986b), they show very mild clinical symptoms which include pyrexia, nervous and respiratory signs.

2.7.2 Demonstration of *T. gondii* in infected tissues

Diagnosis of the infection can be aided by demonstrating the presence of the parasite in host tissue and by the histological appearance of the tissues removed by biopsy or at necropsy (details of the characteristic lesions are described in 2.5). The finding of the rapidly multiplying stage of the parasite, tachyzoites, indicates an active infection is occurring and the presence of tissue cysts may indicate latent infection.

2.7.2.1 Haematoxylin and eosin stain

A rapid but inconclusive diagnosis can be made by microscopic examination of tissue sections stained with haematoxylin and eosin (H&E). Crescent-shaped tachyzoites and spherical tissue cysts can be located in stained sections. However a more specific test is often required as degenerating host cells may resemble degenerating *Toxoplasma* and because of the close morphological similarities with related cyst-producing coccidia like *Sarcocystis* spp. and *Neospora* sp. (Lindsay and Dubey, 1989; Dubey *et al.*, 1989).

2.7.2.2 Immunohistochemical labelling

Conventional histological examination of tissue from infected animals or people is often difficult if the samples are badly decomposed. Moreover, it may be difficult to distinguish *T. gondii* in sections because of the sparse distribution of organisms in the tissues and the close morphology with other related protozoa.

A peroxidase antiperoxidase technique (PAP) adapted to specifically stain microorganisms in tissues has been used to demonstrate *T. gondii* in sections of formalin-fixed, paraffin-embedded tissues from different species (Conley *et al.*, 1981; Uggla *et al.*, 1987). Although *Toxoplasma* antigen can be detected even after one year of fixation in 10% formalin, fixation for shorter periods is recommended.

2.7.2.3 Amplification of specific nucleic acid sequences

Recent advances in molecular biology have devised methods to amplify specific nuclei acid sequences using the PCR technique. This technique has been applied to *T. gondii* by amplification of the B1, p30, TGR1 or the 18S ribosomal DNA (rDNA) targets (Burg *et al.*, 1989; Savva *et al.*, 1990; Dupon *et al.*, 1995). PCR amplification has been successfully used on various clinical specimens from different species (Holliman *et al.*, 1990; Turner and Savva, 1991; Wastling *et al.*, 1993).

Toxoplasma gene amplification has been found to agree closely with mouse inoculation techniques and shows a high degree of specificity (Hitt and Filice, 1992; Wastling *et al.*, 1993), although as a method of diagnosis it is relatively expensive and requires specialized laboratory facilities.

2.7.3 Isolation of *T. gondii*

Isolation of *T. gondii* from infected tissues is considered to be a conclusive and specific test to determine active infection. This is usually achieved by inoculation of tissue samples into laboratory mice. The choice of inoculum (secretions, excretions, body fluids and tissues) will depend upon the case. Cerebral spinal fluid from a child with a possible congenital infection, or lymph node material from a patient with lymphadenopathy or cerebral spinal fluid from a patient with AIDS, can be good sources of *Toxoplasma* parasites. At necropsy, the tissues selected often include brain in TE, cardiac muscle, skeletal muscles and placenta.

Mice are readily infected by intraperitoneal inoculation of tachyzoites or bradyzoites or infected tissue samples. Because other related coccidia like *Neospora* sp. and *Sarcocystis* spp. can appear morphologically similar, isolates should be distinguished by animal inoculation. *T. gondii* has been proved to be highly infective to mice, whereas *Sarcocystis* spp. is not infective and *Neospora* sp. is rarely infective to immunocompetent mice (Dubey *et al.*, 1989; Dubey and Lindsay, 1996). Although

most strains of mice are susceptible to *Toxoplasma* infection, outbred albino female adult mice are recommended when attempting parasite isolation.

Depending on the virulence of the *T. gondii* strain, mice will develop either an acute infection with parasite-rich ascites in 7 to 14 days after inoculation or a chronic infection with presence of bradyzoites within tissues in brain (Derouin *et al.*, 1987). Failure to demonstrate *T. gondii* in mice does not necessarily prove that the mice are not infected. Therefore mice should also be tested for the presence of specific antibodies. Following infection with *T. gondii* mice will develop *Toxoplasma* antibodies by 28 days after infection (Buxton *et al.*, 1979). The experimentally infected mice are monitored for up to 8 weeks after challenge.

Larger samples of tissues can be fed to *Toxoplasma* naive cats which will produce oocysts in their faeces if the tissue samples contains *T. gondii*. Cats are a very sensitive and specific host for the parasite, but are not a very convenient animal model as it is very difficult to obtain *Toxoplasma* naive cats (Dubey and Thulliez, 1993). The faeces are collected for a period of 3 to 15 days after feeding of the test material and are examined for *T. gondii* oocysts microscopically.

2.7.4 Demonstration of specific antibodies in body fluids

Following infection with *T. gondii*, most animals seroconvert. While demonstration of specific *T. gondii* antibodies does not prove active infection, it does establish whether the host has previously been infected with the parasite. Specific serological assays have therefore been a useful aid to diagnosis, being relatively inexpensive and reasonably reliable (Dubey *et al.*, 1987; Arthur and Blewett, 1988; Munday *et al.*, 1987).

Since the development of the "dye test" by Sabin and Feldman in 1948, many serological tests have been described for the detection of antibodies against *T. gondii*. These tests may be divided into those which use the intact parasite as antigen and those which use a fractionated antigen preparation.

In order to be of use the tests must be both specific and sensitive and capable of differentiating between acute and chronic infection, since it has been proved that high antibody levels can still be detected in serum from infected animals years after infection (Dubey and Beattie, 1988). The onset of the infection can be estimated by sequential analysis of samples or by the monitoring of specific IgM antibodies (Remington *et al.*, 1968).

2.7.4.1 Whole parasite antigen

2.7.4.1.1 Dye test

The cytoplasm modifying or "dye test" of Sabin and Feldman (1948) is still considered very reliable. The test has been standardized (Beverley and Beattie, 1958) and adapted to microscale (Feldman and Lamb, 1966; Waldeland, 1976; Balfour *et al.*, 1982).

The test involves mixing live *T. gondii* tachyzoites with the serum sample and complement and adding a methylene blue dye. If there are antibodies to *Toxoplasma* present, the cell wall of the parasite is lysed by the effect of the antibody and complement and cannot take up the dye. The titre reported is the serum dilution at which more than 50% of the parasites remain live and by comparison with a World Health Organization (WHO) international reference serum (Lyng and Simm, 1982), may be expressed in international units per millilitre. Most hosts develop DT antibodies within 4 weeks and titres may remain stable for months or even years.

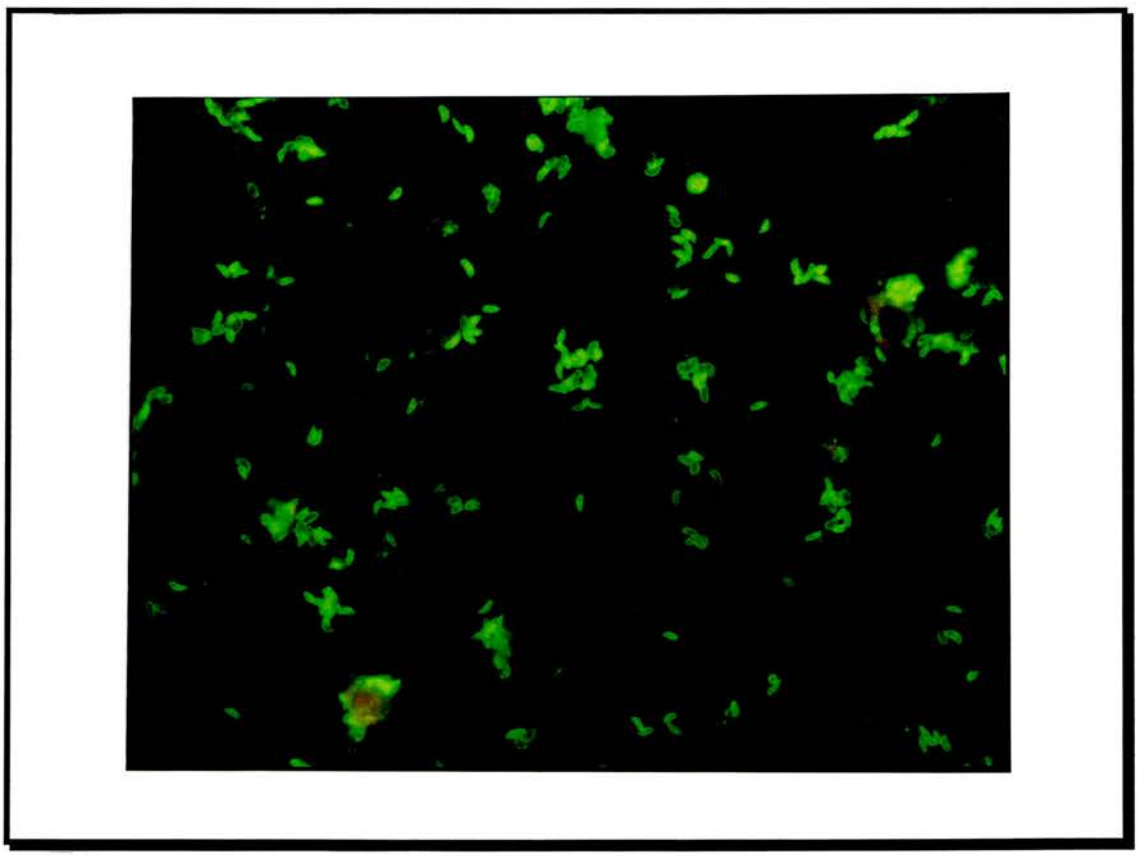
Although the DT is not species specific, a natural globulin in bovine serum can cause false-positive results. This globulin is not inactivated at 56°C for 30 minutes but is inactivated to some extent at 60°C for 1 hour (Dubey, 1986b).

The DT is highly specific and sensitive and is still considered the gold standard by which all other serological test are compared. The main disadvantages of this test are the high cost and the potential hazard of using live organisms.

2.7.4.1.2 Indirect fluorescent antibody test

In this test, whole killed tachyzoites of *T. gondii* (either fixed in formalin or air-dried onto slides) are incubated with serial dilutions of test serum and specific antibody is detected by adding fluorescent-labelled antispecies IgG (or whole immunoglobulin). Numerous workers have found good agreement between the IFAT and the DT (Suzuki *et al.*, 1965; Munday and Corbould, 1971; Munday, 1978; Hughes, 1985).

Figure 2. 9 Whole killed *T. gondii* tachyzoites fixed in formalin and air dried onto slides, incubated with *T. gondii* positive serum and secondary antiserum conjugated with fluorescein iso thiocyanate (FITC) (× 300)



Photograph courtesy of Dr. D. Buxton

A modification of the IFAT was developed for the demonstration of immunoglobulin M *Toxoplasma* antibodies and has proved useful in establishing the

diagnosis of congenital and acute acquired infection in which the presence of IgM signifies an active or recently acquired infection (Remington, 1969).

The drawbacks of this test are that a microscope with ultraviolet (UV) light is required, reading of the results is subjective and false positive responses may occur in individuals with antinuclear antibodies (Araujo *et al.*, 1971) and rheumatoid factor present in their serum (Hughes, 1985).

2.7.4.1.3 Agglutination test

This test was first described by Fulton and Turk (1959) but because of its low specificity and the need for a large number of tachyzoites in each test, it was not a favoured test. The reproducibility and sensitivity of this assay has been increased by growing *Toxoplasma* tachyzoites within mouse sarcoma cells (Desmonts and Remington, 1980).

In this test, formalin-preserved whole parasites are incubated with serum samples at 37° C for 2 hours and at 2°C overnight. The presence of specific IgG antibody is assessed from the nature of the agglutination pattern found. This test is free from cross reactivity with other parasites but is very sensitive to IgM antibodies and these may frequently cause nonspecific agglutination in samples which are negative when tested with other methods (Desmonts and Remington, 1980). This problem can be avoided by treating sera with 2-mercaptoethanol. Titres in this test parallel those in the DT and IFAT, both in human sera and animal sera (Dubey and Beattie, 1988).

The method is accurate, simple to perform, inexpensive and is widely used for screening pregnant women (McCabe and Remington, 1983). It has also been modified to detect IgM antibodies (Dubey and Beattie, 1988).

2.7.4.2 Soluble antigen preparations

In this case the water soluble antigens (both membrane and cytoplasmic antigens) are prepared following water lysis of *Toxoplasma* organisms (Hughes and Balfour, 1981).

2.7.4.2.1 Enzyme-linked immunoabsorbent assay (ELISA)

This technique has become the most widely used test to detect specific *T. gondii* antibodies in many species. This method works on the principle that soluble antigen can be bound to polystyrene or polyvinyl surfaces and that specific antibody bound to the antigen can be detected by an enzyme linked specific antiserum. The resulting colour development is read visually or spectrophotometrically. The ELISA has advantages over the IFAT test of objectivity, quantification and automation and provides quantifiable data (Voller *et al.*, 1976; Buxton *et al.*, 1988). This test uses a crude, sonicated lysate of *T. gondii* tachyzoites as antigen and shows a good correlation with other serological test (Walls *et al.*, 1977; McCabe and Remington, 1983; Buxton *et al.*, 1988; Seefeldt *et al.*, 1989).

2.7.4.2.2 Latex agglutination test

A latex agglutination test (LAT) for use in microtitre plates is commercially available (Eiken Chemical Company®, Japan). In this test, soluble antigen is coated on latex particles. If *Toxoplasma*-specific antibodies are present in the serum to be tested, agglutination takes place. LAT titres from human and animal samples generally correspond with those of DT and IFAT (Wilson *et al.*, 1990). Because this test is simple, easy to perform, inexpensive and does not require any species specific reagent, it has been very useful for screening purposes in different animal species.

2.7.5 Antigen induced cell proliferation

In this test, cultures of primed lymphocytes will proliferate in the presence of specific *T. gondii* antigen quantified by differential uptake of tritiated thymidine

compared to the response in unprimed cells. However, proliferative responses have been observed in some nonimmune individuals, and this may indicate that the assay is not entirely specific (Hughes *et al.*, 1984; Lundén, 1995). Therefore, the significance of antigen specific stimulation of cells can only be evaluated within the context of a wider examination of the immune status of the individual.

2.8 Methods of disease control

2.8.1 Drug treatment

The increasing number of patients with TE due to the AIDS pandemic, has stimulated much interest in new effective drugs against *T. gondii* (Araujo and Remington, 1992). Current drugs are only active against the tachyzoite stage of *T. gondii*, however the majority of cases of TE in immunocompromised patients result from a reactivation of a previous latent infection, characterised by the presence of bradyzoites within the resistant tissue cysts. There are few effective drugs currently available that are effective against *T. gondii* tissue cysts.

This section will discuss some of the chemotherapeutic agents with demonstrable *in vivo* activity against *T. gondii.*

2.8.1.1 Drug therapy in human toxoplasmosis

2.8.1.1.1 Inhibitors of dihydrofolate reductase (DHFR)

These substances have been investigated because *T. gondii* is dependent on the host for purines (Schwab *et al.*, 1995).

Pyrimethamine is a widely used therapy for toxoplasmosis and has been administered in combination with other agents, such as sulfadiazine. These two drugs

are the combination of choice for patients with TE (Fung and Kirschenbaum, 1996). They act synergistically by blocking the metabolic pathway involving paraminobenzoic acid and the folic-folinic acid cycle.

Trimethoprim is active against *T. gondii* but less so than pyrimethamine.

Trimetrexate and piritrexim are two of the inhibitors of the DHFR with activity against *T. gondii* and significant protection against *Toxoplasma* infection (Araujo and Remington, 1992).

2.8.1.1.2 Sulfonamides

These substances, namely sulfadiazine, sulfamethazine, sulfamerazine, act against *T. gondii* by inhibiting the dyhidrofolic acid synthetase. Thus interfering with the parasite's ability to use paramonobenzoic acid. As mentioned before, these substances are widely used in combination with pyrimethamine. They are generally well tolerated although sometimes thrombocytopenia and/or leukopenia may develop (Fung and Kirschenbaum, 1996).

2.8.1.1.3 Clindamycin

In experimental models of acute and chronic infection, treatment with clindamycin has been shown to reduce mortality as well as reduce tachyzoite numbers and inflammation of the brain. The combination of pyrimethamine and clindamycin was comparable to the widely used pyrimethamine-sulfadiazine for the treatment of TE in AIDS (Remington and Vildé, 1991).

2.8.1.1.4 Macrolides and azalides

Roxithromycin, a derivative of erythromycin, has been reported to protect mice against lethal infection with *T. gondii*. Azithromycin, has been proved to be

more active than other macrolides, although the protection depends on the strain of *T. gondii* used (Araujo and Remington, 1992).

2.8.1.1.5 Tetracyclines

Both doxycicline and minocycline have *in vitro* and *in vivo* activity against the infection in mice, although their mechanism of action is unknown for the moment (Wong and Remington, 1993).

2.8.1.1.6 Hydroxynapthoquinones

The hydroxynapthoquinone, atovaquone, has a potent activity against both the tachyzoite and tissue cysts of *T. gondii* (Huskinson-Mark *et al.*, 1991). In murine experimental models, atovaquone has significantly reduced mortality (Wong and Remington, 1993).

2.8.1.1.7 Spiramycin

It has been shown that early treatment with spiramicyn may prevent transmission of infection to the foetus but most probably cannot interrupt an existing brain infection, which is the most severe outcome of congenital toxoplasmosis in humans (Schoondermark *et al.*, 1993).

2.8.1.1.8 Others

Several other agents have been experimentally tested for activity against the parasite. Eimycine an inhibitor of DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) synthesis; arprinocid which inhibits hypoxantine transmembrane transport and dapsone an inhibitor of *T. gondii* dihydropteroate syntheses.

2.8.1.2 Drug therapy in sheep

2.8.1.2.1 Monensin

The anticoccidial monensin was the first chemoprophylactic agent shown to be effective against toxoplasmosis. When fed to the ewes daily during pregnancy at a rate of 15mg per animal, treatment with monensin would control a subsequent infection with *T. gondii* (Buxton *et al.*, 1987; Buxton *et al.*, 1988).

2.8.1.2.2 Decoquinate

The anticcocidial drug decoquinate fed daily to pregnant ewes (2mg per Kg bodyweight) can also significantly reduce the effect of a *Toxoplasma* oocyst challenge during pregnancy (Buxton *et al.*, 1996).

2.8.1.2.3 Pyrimethamine and sulphamethazine

Buxton and coworkers (1993a) tested an effective treatment in human medicine (pyrimethamine and sulphamezathine) to treat *Toxoplasma* infection in sheep after infection had become established within the placenta and foetus. The results showed that this combination suppressed the parasite in sheep, with no lambing losses in the treated ewes.

2.8.1.2.4 Baquiloprim and sulphadimidine

This combination of agents which also blocks the folate synthesis has given promising results in a controlled pilot study in non pregnant sheep (Buxton, 1993a).

2.8.2 Vaccination against *T. gondii* infection

Unlike many other parasitic diseases, infection with *T. gondii* induces a life-long protective immunity and therefore offers the possibility of controlling the disease by vaccination.

It is generally agreed that a vaccine to prevent oocyst shedding in cats would be very desirable to prevent *T. gondii* oocyst shedding. However, this would only be practical if all the cats would be vaccinated. This is extremely difficult because of the stray and wild cat population. Currently there is a vaccine available in the USA to prevent oocyst shedding in cats. This is a live vaccine which involves a strain of *T. gondii* bradyzoites unable to form oocysts in the gut of the cat (Toxoplasma-263) (Frenkel *et al.*, 1991). However, because it is a live vaccine and has to be grown in mice, the production is problematic and it has a very short self life.

A more realistic aim would be the prevention of abortion both in woman and ewes and prevention of tissue cysts formation in intermediate hosts. An effective vaccine to reduce tissue cysts would be of great benefit in reducing transmission of the disease to people via consumption of tissue cysts from infected animals.

A potential vaccine must afford long-term protection, be safe, cost-effective and easy to deliver.

2.8.2.1 Live vaccines

Temperature-sensitive (Ts) mutants of the RH strain have been recently described (Pfefferkorn and Pfefferkorn, 1976). One of these, which grew normally at 37°C but persisted for only 40 hours at 38°C and 39°C and 20 hours at 40°C, was shown to be non-lethal to mice and hamsters. Organisms were not isolated from infected mice two months after the initial inoculation and an effective and sustained immunity developed (Waldeland and Frenkel, 1983; McLeod *et al.*, 1988; Suzuki and Remington, 1990). The Ts-4 mutant would appear to combine the advantages of both live and killed vaccines, with its ability to proliferate for only a short period *in*

vivo and to stimulate an effective CMI response. Because the Ts-4 mutant is a live vaccine, it is not suitable for use in people.

In the USA, a vaccine to prevent cyst formation in pigs is being tried using RH strain of *T. gondii.* Parasites of this strain are highly virulent in mice but cause only mild disease in pigs and the parasites do not persist as tissue cysts in the animals. When pigs that recover from the infection are re-infected with parasites of cyst-forming strains, they do not show any signs of disease and have fewer cysts in their tissues (Dubey *et al.*, 1991).

In 1988, a live vaccine for the control of toxoplasmosis was launched on the market in New Zealand (O'Connell *et al.*, 1988; Wilkins *et al.*, 1988) and in 1992 in the United Kingdom and Eire (Buxton, 1993b). The vaccine comprises tachyzoites of the S48 strain of *T. gondii* which is a short-lived, incomplete strain of the parasite that exists only as the tachyzoite stage and is unable to form tissue cysts (Wilkins and O'Connell, 1992). S48 *T. gondii* was originally isolated from an ovine abortion in New Zealand and then passaged in laboratory mice about 3000 times before it was shown to have lost the ability to develop bradyzoites in tissue cysts and initiate the sexual life cycle of the parasite in cats (Buxton and Innes, 1995). When administered to sheep by subcutaneous injection, S48 tachyzoites afforded a high degree of protection against *Toxoplasma* induced abortion following challenge with oocysts (O'Conell *et al.*, 1988; Buxton *et al.*, 1991). Live S48 tachyzoites now form the basis of the first commercially available veterinary vaccine for toxoplasmosis (Buxton and Innes, 1995). However, this vaccine is potentially hazardous for man and has a short shelf-life making commercial production and distribution problematic.

2.8.2.2 Killed vaccines

Several attempts to induce protective immunity in sheep by inoculation of whole killed *Toxoplasma* parasite preparations have been unsuccessful (Beverley *et al.*, 1971a, b; Wilkins *et al.*, 1987; Buxton *et al.*, 1989), and although many live lambs were born, placental and fetal infection were not eliminated.

Toxoplasma surface antigens combined with Quil A (immunostimulating complexes or ISCOM) have been shown to elicit good humoral and cellular immune responses and to provide protection against a lethal challenge in mice (Uggla *et al.*, 1988). However, while good titres of antibody were stimulated by the vaccine, only moderate protection with reduction in abortion rate (not statistically significant) was detected when the same preparations were use to prevent congenital infection and abortion in pregnant sheep (Øvernes *et al.*, 1991; Lundén, 1995).

Good protection against acute infection in mice has been observed after immunization with the major surface protein of *T. gondii* tachyzoites, P30 or SAG1, either incorporated into liposomes (Bülow and Boothroyd, 1991) or in conjunction with Quil A (Khan *et al.*, 1991).

The search for an effective and safe vaccine against toxoplasmosis continues. The main problem in using killed vaccines is the difficulties of inducing an appropriate cell mediated immune response. Therefore there has been much interest in studying different routes of antigen delivery to the host. Mucosal immunity (IgA and CMI) can be elicited following oral *T. gondii* infection (Chardès *et al.*, 1990; Huskinson *et al.*, 1990; Chardès and Bout, 1993). Vaccines are commonly administered by a parenteral route, but the mucosal route is attractive for both logistical and conceptual reasons. Professor Bout and colleagues in Tours (France) have shown that mucosal vaccination of mice against toxoplasmosis using SAG1 antigen and cholera toxin (CT) as an adjuvant was very effective (Chardès *et al.*, 1993).

Nasal vaccination against *T. gondii* represents a novel approach to vaccination against infectious diseases and has the advantage over oral administration that the degradation of the antigen is minimal. A vaccine using killed antigenic material, administrated nasally and effective after one dose which would induce both mucosal and systemic immune responses against *T. gondii* infection and would be very desirable (Bourguin *et al.*, 1993; Chardès *et al.*, 1993).

Preliminary studies have shown that the native 30 kDa protein SAG1 of *T. gondii* coupled to CT has a great potential as a protective vaccine, affording 85%

protection in mice (Darcy *et al.*, 1992; Bourguin *et al.*, 1993). However it remains to be seen whether such a vaccine preparation would afford any protection against congenital toxoplasmosis and abortion in sheep.

As recently stated by the WHO Veterinary Public Health Working group on Toxoplasmosis Vaccine Development and Technology, there is a need for a vaccine for meat producing animals. The general aims of such vaccine development are to reduce abortion and neonatal mortality in sheep and to reduce the number of tissue cysts in meat for human consumption and in this way reduce disease transmission to people. Successful development of a killed vaccine for use in sheep would permit its wider use and bring a vaccine for the prevention of human toxoplasmosis very much closer.

2.8.3 Management practices to reduce *T. gondii* infection

There are two main targets in the control of toxoplasmosis. The first is prevention of congenital *Toxoplasma* infection and the second is to reduce the risk of transmission of *T. gondii* through the consumption of infected meat.

2.8.3.1 Oocyst infection

To prevent infection in cats and shedding of oocysts in cats faeces, control programs should attempt to reduce infection of cats. When young cats start hunting for the first time they eat *T. gondii* infected small rodents and birds. Following a primary infection with *T. gondii* the cats will excrete high numbers of oocysts which can persist in the environment for up to one year (Dubey and Frenkel, 1972). The risk for contamination of animal feeds is high when litters of cats inhabit and defecate in hay, grain or other feed store areas of the farm. Fecal contamination of pasture areas can also be a significant cause of infection in grazing livestock.

Elimination of cats from farm areas is very difficult, therefore it is advised that where possible feed should be stored in cat-proof areas, particularly feed for

pregnant sheep. Cats should never be fed undercooked meat, viscera or bones. Moreover, dead animals should be removed promptly from the farm area to prevent scavenging by cats and foetal membranes and dead foetuses should be buried or incinerated to prevent infection of felids and other animals on the farm.

Because oocysts can survive in the environment for several months up to one year, contact with sporulated oocysts is likely to occur most frequently when working with soil or when drinking contaminated water. Although human infection occurs commonly by ingestion of tissue cysts in infected meat, to avoid oocysts infection, individuals at high risk should avoid feeding pet cats undercooked meat, prevent the cat hunting as far as possible, clean the litter box daily with scalding water and incinerate or flush the faeces, wear gloves when working with soil and keep sand pits covered.

2.8.3.2 Consumption of T. gondii *cysts*

People are commonly infected following the ingestion of tissue cysts present in undercooked infected meat. Veterinarians, farm workers, especially if pregnant, and AIDS patients, should take precautions to avoid infection when handling raw meat. Meat should be cooked to at least 66°C for 20 minutes (Dubey *et al.*, 1990). Hands should be cleansed thoroughly after handling raw meat. Freezing meat at -20°C greatly reduces the viability of tissue cysts (Dubey, 1988). Microwaving does not kill all *T. gondii* because of uneven cooking (Lundén and Uggla, 1992).

2.9 Neospora caninum and neosporosis

Neospora caninum is a recently recognised protozoan parasite of livestock and companion animals. The disease was first recognised in Norwegian dogs with encephalomyelitis and myositis (Bjerkås *et al.*, 1984). The new genus, *Neospora* was not proposed until 1988 following isolation of the parasite into tissue culture (Dubey *et al.*, 1988a).

Neosporosis has been described as a major cause of abortion in cattle in many countries and an important cause of neuromuscular paralysis in dogs. *Neospora caninum* is very closely related to *T. gondii* and unlike *T. gondii* is a major cause of disease in cattle. Therefore a brief summary of current knowledge of the parasite and the disease it causes in cattle may be pertinent to the understanding of *T. gondii* infection in cattle.

2.9.1 Neosporosis in cattle

Thilsted and Dubey (1989) first reported *N. caninum*-like organisms in the brain of aborted bovine foetuses. Toxoplasmosis was excluded as the cause of abortion because aborting cows did not show *Toxoplasma*-specific antibodies and *T. gondii* is not recognised as abortifacient in cattle.

Abortion is the only clinical sign observed in infected cows (Anderson *et al.*, 1991). Dairy and beef cows of any age (Anderson *et al.*, 1991; Wouda *et al.*, 1995) may abort from 3 months of gestation to term. Foetuses may die in the uterus, be resorbed, mummified, autolysed, stillborn, born alive, but diseased, or born clinically normal but chronically infected. There have been reports on abortions occurring sporadically or in groups within a few weeks or abortions persisting in a herd (Dubey and Lindsay, 1996). *Neospora caninum* seems to be able to cause repeated abortion in the same cow although the rate of repeat abortion in low (<5%) (Obendorf *et al.*, 1995; Anderson *et al.*, 1995). Degenerative and inflammatory lesions are most common in CNS, heart, skeletal muscle and liver of the foetus (Wouda *et al.*, 1996). Examination of maternal and foetal sera for *Neospora* specific antibodies can aid diagnosis, which is based mainly on detection of the parasite by immunohistology of infected tissues. Neosporosis in congenitally infected calves is mainly characterised by neurologic signs (Parish *et al.*, 1987).

2.9.2 Life cycle and transmission

Neospora caninum has been found in dogs, cattle, sheep, goats, horses and deer (reviewed by Dubey and Lindsay, 1996). The complete life cycle of *N. caninum* is not well understood and as yet the definitive host remains to be determined. Transplacental infection, which may occur repeatedly in the same animal (Barr *et al.*, 1993), is the only natural route of transmission to date. *Neospora caninum* is infective by experimental subcutaneous, intraperitoneal, intramuscular, intravenous and oral routes (reviewed by Dubey and Lindsay, 1996). Bradyzoites within the tissue cysts are resistant to HCl-pepsin solution, which may indicate that like *T. gondii* carnivorism is part of the life cycle (Lindsay and Dubey, 1990).

2.9.3 Immunity to *N. caninum*

While the host immune response to *T. gondii* has been well studied, little is known regarding the induction of the immune response following infection with *N. caninum*. Seroconversion in cattle has been demonstrated following experimental and natural infection with *Neospora* (Conrad *et al.*, 1993). Detection of *Neospora*-specific antibodies in the sera of cows by both IFAT (Dubey *et al.*, 1988b) and ELISA (Björkman *et al.*, 1997; Williams *et al.*, 1997), can be useful in the diagnosis of bovine abortion and to study the seroepidemiology of the disease. Although morphologically *Toxoplasma* and *Neospora* are very similar, they are genetically distinct (Marsh *et al.*, 1995). Moreover, there are antigenic differences between these two parasites, demonstrated by the lack of cross reactivity between *N. caninum* and *T. gondii* when examining specific antibodies. *Neospora* lack both of the major surface proteins of *T. gondii* namely SAG1 (P30) and SAG2 (P22) (Brindley *et al.*, 1993). Little is known regarding the induction of the cellular immune response during *Neospora* infection. It has been reported that IFNγ may be involved in the host immunity as the cytokine is able to inhibit intracellular multiplication of *N. caninum*

in vitro (Innes *et al.* 1995c). Recent studies on experimentally infected mice have demonstrated a cellular response to *N. caninum*. It can be characterised by the induction of T cells and is mediated by the production of IL-12 and IFNγ as the administration of antibodies to these cytokines reversed natural protection in mice (Khan *et al.*, 1997).

Chapter 3

Materials and Methods

3. Materials and Methods

3.1 Animals

All the animals involved in this study were kept in an environment considered to offer minimal risk of *T. gondii* infection.

3.1.1 Sheep (*Ovis auries*)

Twenty-two sheep seronegative by ELISA for antibodies to *T. gondii* were infected orally with different doses of *T. gondii* oocysts.

Figure 3. 1 Welsh Mountain ewe and lamb (*Ovis auries*)

Photograph courtesy of Dr. D. Buxton

3.1.2 Cattle (*Bos taurus*)

Ten cattle seronegative by IFAT for antibodies to *T. gondii* and *Neospora caninum* were orally infected with two doses of *T. gondii* oocysts.

Figure 3. 2 Young Ayrshire bulls (*Bos taurus*)

Photograph courtesy of Mr. J. McCracken

3.1.3 Mice (*Mus musculus*)

Mature female Swiss White mice (inbred) were used to passage and maintain *T. gondii* tachyzoites of the S48 strain as well as to study the viability of *T. gondii* cysts in tissues. Females were used to avoid fighting and tail biting among competing male mice. Porton mice were used to maintain the M3 isolate of *T. gondii*.

Figure 3. 3 Strain Porton (*Mus musculus*)

Photograph courtesy of Mr. S. Wright

3.1.4 Cats (*Felis catus*)

Cats of both sexes, under six-months of age and seronegative for antibodies to *T. gondii,* were obtained from a *Toxoplasma* free colony in the University of Newcastle (UK).They were used to obtain viable *T. gondii* oocysts of the M3 isolate.

Figure 3. 4 ***Felis catus***

Photograph courtesy of Mr. B. Easter

3.2 *Toxoplasma gondii*

3.2.1 M3 isolate

3.2.1.1 Maintenance

The complete M3 isolate of *T. gondii* was originally isolated from the brain of a stillborn lamb at Moredun Research Institute (Edinburgh, UK) on the 25th of April 1986, following i.p. inoculation of 2 female Swiss White mice with foetal tissue. It was passaged for the first time 3 months later, and maintained by once yearly passage in Porton mice (minimally inbred). The brains were aseptically removed and homogenized in Hank's balanced salt solution (HBSS) by passage through a 16 gauge (G) needle. Between 40-100 tissue cysts were inoculated i.p.

The M3 isolate was chosen for this project because it has been proved to be a complete isolate, capable of causing clinical toxoplasmosis in sheep and bradyzoites of this isolate can induce oocysts formation in seronegative cats. This isolate was experimentally used for the first time in 1991 (Buxton *et al.*, 1991).

3.2.1.2 M3 tissue cysts

Brains from chronically infected Porton mice (4 on average) were removed and homogenized in an equal volume of phosphate buffered saline (PBS), using an 18G needle and a syringe. Suspensions containing an estimate 10^3 *T. gondii* tissue cysts were prepared.

3.2.1.3 Oocysts

Cats used as *T. gondii* donors were maintained in a negative pressure isolator to minimize contamination of the environment with infective oocysts

After a short period of acclimatization, each cat was infected with approximately 10^3 M3 *T. gondii* tissue cysts, mixed with a little food. Faeces were collected daily and screened for the presence of oocysts. Oocyst shedding commences approximately 5 days post infection, and continues for 7-10 days. After a 3 day period when no oocysts were detected in the faeces, the animals were removed from the isolator, which was then sterilized using a peroxide based disinfectant.

Cats were fed using a proprietary tinned food, and water was available *ad-lib*. Moist, oily food was given in the event the animal became constipated (a not infrequent occurrence during *T. gondii* infection in cats). Oocysts delayed in the gut of the animal due to constipation usually fail to sporulate, and so are useless for infection studies.

Sporulated *T. gondii* oocysts of the M3 isolate were obtained from infected cat faeces using the Saturated Salt Flotation Technique described by Buxton *et al.* (1988). Briefly, a small amount (0.5 to 1g) of infected faeces was suspended in 10ml of water and then centrifuged for 5 minutes at 1000*g*. The pellet was resuspended in 10ml of saturated sodium chloride (NaCl) and the mixture centrifuged at 1000*g* for other 5 minutes. The meniscus was removed and transferred to an improved Neubauer haemocytometer for microscopic examination (Vickers M75, x40 objective) for the presence of oocysts. If the sample contained oocysts the remainder of the faeces was processed in similar manner.

Faeces were diluted 1:10 in water, homogenized thoroughly and then the suspension was sieved through 1mm mesh. Aliquots of 50ml were centrifuged for 10 minutes at 1000*g* (relative centrifugal force), the supernatant was discarded, and the pellet resuspended in 50ml of saturated NaCl. After 10 minute centrifugation at 1000*g*, the meniscus was removed (approximately 5ml) and diluted 1:10 with water and then centrifuged for 10 minutes at 1000*g*. The pellet was resuspended in small volume of water and counted using an improved Neubauer haemocytometer. Oocysts were allowed to sporulate at room temperature for 3 days before storage in 2% sulfuric acid (H_2SO_4).

3.2.1.4 Inoculum

Inoculum for sheep and cattle were made up by serial dilution, administered as a 2ml aliquot by gavage, and washed down with 50ml water.

3.2.2 S48 strain

T. gondii strain S48 was originally isolated at Wallaceville (New Zealand) from an aborted sheep foetus (Hartley and Marshall, 1957). This strain was passaged twice-weekly in laboratory mice, about 3000 times before being shown to have lost the ability to develop bradyzoites (Wilkins *et al.*, 1987). The S48 strain has lost its capacity to form oocysts in cats and is, therefore, referred to as an incomplete strain (Wilkins *et al.*, 1987; Frenkel *et al.*, 1986). These parasites were used to prepare antigen for the Antigen induced proliferation assay on peripheral blood mononuclear cells and for the IFAT.

3.2.2.1 Maintenance

T. gondii tachyzoites of the S48 strain were maintained by passage in female Swiss White mice. Mice were killed with carbon dioxide (CO_2) and parasites were harvested with a 26 G needle and syringe from the peritoneal cavities of mice that had been infected three days earlier with 10^6 tachyzoites by i.p. injection (cellular contamination below 2%).

3.2.3 RH strain

T. gondii complete strain RH was originally isolated at Cincinnati (USA) from mice inoculated with a tissue sample from the CNS of a 6 year old boy with a fatal acute nonsupurative encephalitis (Sabin, 1941). These parasites were used to prepare antigen for the ELISA.

3.2.3.1 Maintenance

T. gondii tachyzoites of the RH strain were maintained by passage in female Swiss White mice. Mice were killed with CO_2 and parasites were harvested with a 26G needle and syringe from the peritoneal cavities of mice that had been infected three days earlier with 10^6 tachyzoites by i.p. injection (cellular contamination below 2%).

3.3 Antigen preparation

3.3.1 Water soluble fraction of *T. gondii* antigen

The water soluble fraction of both *T. gondii* strains, RH and S48, antigen was obtained based on the method described by Voller *et al.* (1976). RH strain was used for the ELISA and S48 strain for the antigen induced proliferation assay on peripheral blood mononuclear cells described later.

RH and S48 strains of *Toxoplasma* tachyzoites were obtained as described previously (3.2.2 and 3.2.3). The peritoneal washings collected from infected mice were given a minimum of six alternate 15 minute washes in 0.3M PBS and HBSS (pH 7.4) to remove the host cell contamination to below 2%. After the final wash, the supernatant was discarded and the pellet containing the tachyzoites was lysed by resuspension in distilled water (10 times its volume). In order to rupture the tachyzoites, the pellet was freeze-thaw three times followed by 6×30 seconds cycles of sonication at 4°C. Particulate matter was then removed by centrifugation at 10000*g* for 30 minutes at 4°C and the water soluble extract (supernatant fluid) was stored in aliquots of 50µg/ml at -20°C until required. Protein concentration was assayed using BCA reagent (Sigma Chemical, UK) and read using a spectrophotometer.

3.3.2 Whole killed *T. gondii* antigen

T. gondii antigen using the S48 strain was prepared by modification of the method described by Goldman (1957) and Munday and Corbould (1971) to be used in the IFAT.

T. gondii tachyzoites of the S48 strain were harvested from mice infected 3 or 4 days previously by the peritoneal route (see 3.2.2). The peritoneal washings collected were given a minimum of six alternate 15 minute washes in 0.3M PBS and HBSS (pH 7.4) to remove the host cell contamination to below 2%. Suspensions of formalinised tachyzoites (Formaldehyde solution 37/40%w/v, with 10-14% methanol added as stabilizer, FISONS®) (10^7per ml) (0.2%v/v) were prepared and stored at -20°C until required.

3.4 Sample collection

3.4.1 Serum samples

Blood samples were taken periodically from the jugular vein of sheep and cattle (see Chapter 4 and Chapter 6 for further details) into preservative-free evacuated blood collection tubes, Vacutainer®(Becton Dickinson Ltd., UK) and allowed to clot. Following retraction of the clot, the serum was obtained after centrifugation at 500*g* for 15 minutes and then stored at -20° C until required.

Blood samples were taken from the tail of Swiss White mice, 8 weeks after inoculation with tissue samples from sheep and cattle, into heparinized haematocrit tubes (Harsaw Chemicals Ltd., UK). The serum was obtained by centrifugation for 10 minutes and then stored at -20°C until required.

3.4.2 Cell free peripheral blood plasma

Blood samples were taken periodically from the jugular vein of sheep and cattle (see Chapter 7 for further details) into preservative-free heparinized (10^3U/ml of Heparin sodium salt, Sigma Chemical, UK) evacuated blood collection tubes, Vacutainer®(Becton Dickinson Ltd., UK) and centrifuged at 400 *g* for 20 minutes and the plasma removed and stored at -20°C until required.

3.4.3 Peripheral blood mononuclear cells (PBMC)

Blood samples were taken periodically from the jugular vein of sheep and cattle (see Chapter 7 for further details) into preservative-free heparinized (10^3U/ml of Heparin sodium salt, Sigma Chemical, UK) evacuated blood collection tubes, Vacutainer®(Becton Dickinson Ltd., UK). Ten ml of each sample were mixed with 10ml of sterile PBS and placed into a sterile 20ml Universal, centrifuged at 400*g* for 20 minutes with the brake off in a GS-6R Beckman centrifuge® and the buffy coat removed to a sterile 20ml Universal. The PBM cells were resuspended in HBSS supplemented with 2% fetal bovine serum (FBS), 100IU/ml of penicillin, 100µg/ml of streptomycin (Northumbria Biologicals, UK), gently layered onto Lymphoprep® (Nycomed, Norway) in a 10ml conical centrifuge tube and centrifuged at 500*g* for 40 minutes in a GS-6R Beckman centrifuge® with the brake off. PBM cells from the interface were then collected in a 20ml Universal® and washed twice with HBSS supplemented with 2% FBS, 100IU/ml of penicillin and 100µg/ml of streptomycin.

3.4.3.1 Isolation of peripheral blood mononuclear cells for Polymerase Chain Reaction

The peripheral blood mononuclear cells obtained as described in 3.4.3, were placed into a 1.5ml UV sterilized eppendorf. The remaining red blood cells (RBC) were lysed by the addition of 1ml of 10mM Tris ammonium chloride followed by centrifugation at 13000*g* for 30 seconds. The cells were then washed three times by

resuspension in PBS and centrifugation at 13000*g* for 30 seconds. The cell pellet was stored at -20°C until ready for use.

3.4.3.2 Peripheral blood mononuclear cells for antigen induced proliferation assay

The peripheral blood mononuclear cells obtained as described in 3.4.3, were resuspended at 4×10^6 cells/ml in culture medium comprising Iscove's Modified Dulbecco's medium (IMDM) (Gibco, UK) supplemented with 10% FBS, 100IU/ml of penicillin, 100µg/ml of streptomycin and 5×10^{-5} M 2-Mercaptoethanol (Sigma Chemical, UK).

3.4.3.3 Peripheral blood mononuclear cells for phenotypic analysis

Peripheral blood mononuclear cells were collected as described in 3.4.3.2 and aliquots of 2×10^6 cells per monoclonal antibody were used.

3.4.4 Cytokine analysis

Supernatants from short term cultures of PBM cells were collected after 96 hours of stimulation with specific antigen and stored at -20°C until required (see Chapter 7 for further details).

3.4.5 Tissue samples

Either 6 weeks or 6 months after challenge of sheep and cattle postmortem examinations were carried out. Carcasses of the killed animals were first inspected for external lesions and then opened for gross examination of the internal organs. During the postmortem examination, tissues were collected for histopathological

examination, bioassays in mice and PCR (see Chapter 4 and Chapter 5 for further details).

Tissue samples for histopathological examination were fixed in 10% formal saline (40% formaldehyde and 0.9% sodium chloride) until processed. The samples to be inoculated in mice were collected aseptically and those analyzed by PCR were collected in sterile conditions and stored at -20°C until required.

3.5 Clinical response

3.5.1 Rectal temperatures

Rectal temperatures of sheep and cattle were recorded daily for the 3 days before and 14 days after oral dosing with a clinical electronic thermometer (Solex, UK).

3.5.2 Detection of parasitaemia by B1 Polymerase Chain Reaction

3.5.2.1 Detection of T. gondii *DNA in PBM cells by the B1 PCR*

Peripheral blood mononuclear cells were analysed by the B1 PCR to detect and amplify *T. gondii* DNA following the procedure described by Wastling and collaborators (1993). Method modified from that of Burg and co-workers (1989).

3.5.2.1.1 Peripheral blood mononuclear cell preparation

The peripheral blood mononuclear cells were obtained as described in 3.4.3.1

3.5.2.1.2 DNA extraction from peripheral blood mononuclear cells

The cell pellet was defrosted and resuspended in K buffer containing proteinase K (100 µg/ml) (Sigma Chemical, UK) and Tween 20 (polyoxyethilene (20) sorbitan monosaturate) (Fison, UK) (0.5%). After incubating the mixture at 55°C for 1 hour, proteinase K was inactivated by boiling and the samples were stored at -20°C.

3.5.2.1.3 Preparation of *T. gondii* tachyzoites of the S48 strain

Toxoplasma tachyzoites were passaged in Swiss White mice every three days by intraperitoneal inoculation (10^6 tachyzoites)as described in 3.2.2. Peritoneal exudate was removed and the parasite cleaned by washing with PBS followed by HBSS and then centrifuged at 500g for 5 minutes. Parasites were counted using a haemocytometer and preparations containing 98% tachyzoite suspension were used.

3.5.2.1.4 *T. gondii* DNA extraction for use as a positive control

DNA was extracted from 10^9 *T. gondii* tachyzoites for use as positive control material. Parasites were lysed in 50mM Tris (pH 8.0), 50mM EDTA, 1% sodium dodecyl sulphate (SDS), containing 100µg/ml proteinase K. After incubation at 50°C for three hours, the nucleic acid was extracted with an equal volume of phenol/chloroform followed by an equal volume of chloroform. DNA was then precipitated with 2 volumes of ethanol (100%) and 0.1 volume of 3M sodium acetate at -20°C for 1 hour, followed by centrifugation at 10000 g. The pellet was washed in 70% ethanol and then resuspended in Tris-EDTA solution (10mM Tris, EDTA, pH 7.6) and the DNA quantified by measuring UV absorption at 260 nm.

3.5.2.1.5 Quantification of *T. gondii* DNA

The concentration of DNA preparations was assessed by taking readings of Optical Density $(OD)_{260}$ using a spectrophotometer (OD_{260} equal to 1 corresponds to 50μg/ml double stranded DNA). Readings were also taken at OD_{280}. DNA preparations free from protein possessed an OD_{260}: OD_{280} ratio approaching 2.0.

3.5.2.1.6 The B1 polymerase chain reaction

The polymerase chain reaction was carried out in a thermal cycler (Hybaid Ltd., UK) over 25 cycles in 50μl volumes which were overlaid with 30μl of mineral oil. The B1 PCR system required the use of nested primers (see Figure 3. 5).

The method of Burg *et al.* (1989) was modified to amplify the multi-copy B1 gene. The reaction mixture contained 10mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 40mM KCl, 0.01% gelatin, 0.1mM d-NTPs (deoxy nucleotide triphosphate) (Pharmacia Biosystem Ltd., UK), 0.2μM of each oligonucleotide primer (Oswell DNA Services, Edinburgh, UK) and 2.5 units of *Taq* DNA polymerase (Boehringer Mannheim, Germany). Primers P1, P3 and P4 were as described by Burg *et al.*, (1989) but P2 consisted of only 20 bases corresponding to nucleotides 757-776 of the B1 gene.

Figure 3. 5 Primers for detection of the multi-copy gene B1

P1	(external), annealing to nucleotides 694-714	**GGA ACT GCA TCC GTT CAT GAG**
P2	(internal), annealing to nucleotides 757-776	**TGC ATA GGT TGC AGT CAC TC**
P3	(internal), annealing to nucleotides 853-831	**GGC GAC CAA TCT GCG AAT ACA CC**
P4	(external), annealing to nucleotides 887-868	**TCT TTA AAG CGT TCG TGG**

Amplification was performed with P1 and P4 to give a 193 base pair (bp) product over 25 cycles of denaturation at 93°C for 20 seconds, annealing at 50°C for 20 seconds and extension at 70°C for 40 seconds. The final cycle was extended by 4 minutes at 70°C. Amplification products were then diluted 1: 20 in distilled water to reduce amplification of non-specific products and amplified again with nested primers P2 and P3 over 25 cycles in the same conditions to give a 96 bp product. With this method, 0.05 pg of DNA could be seen on 2% agarose gel.

Negative controls consisting of distilled water were run for each set of tests to monitor cross-contamination.

A DNA ladder of 50-1000 bp (Amersham, UK) with a concentration in each band of approximately 50ng/5µl of DNA ladder loaded, was used to estimate the band size of the amplified product.

3.5.2.1.7 Agarose electrophoresis of DNA

Amplification products were analyzed on 2% agarose gels (w/v) (nucleic acid grade ultrapure agarose, Bio-Rad Laboratories Ltd., UK) in Loening E buffer (900mM Tris, 750mM NaH_2PO_4, 25mM EDTA, pH 7.6-7.8) using agarose "sub-cell" gel tanks (Pharmacia, UK). Ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide; homidium bromide; 500µ/ml, Sigma Chemical, UK) was added to molten agarose at a final concentration of 0.2 µg per ml. DNA loading buffer (0.1% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 75mM EDTA, 25% sucrose) was added 1:9 (v/v) to samples prior to loading, and electrophoresis was carried out at 60 volts per cm in Loening E buffer until required resolution was achieved. DNA was then visualized using ultraviolet translumination on a Hybaid Crosslinker (Hybaid, UK).

The detected product was confirmed to be *T. gondii* DNA by transfer of the agarose gel to nitrocellulose membrane and detection of bound digoxigenin (DGI) labelled probes to *T. gondii* DNA.

3.5.2.1.8 Southern Blotting of DNA to nitrocellulose membrane

DNA was then transferred from agarose gel to nitrocellulose membranes using a modification of the method described by Smith and Summers (1980). The gel was denaturated in 0.5M NaOH, 1.5M NaCl for 30 minutes, and then neutralized in 1.5M ammonium acetate, 0.02M NaOH for 30 minutes. The membrane was then placed on top of the gel followed by 3 sheets of Whatman 3MM chromatography paper (Whatman, UK) soaked previously in neutralizing solution for 20 minutes (1.5M NH_4 acetate, 0.02M N_aOH). Finally a 3cm stack of paper towels and 1Kg weight were placed on top of the membrane. Transfer was carried out over 3 to 18 hours. The filter was then washed in neutralizing solution for 20 minutes, blot dried with 3MM paper and incubate at 37°C for 10 minutes. The DNA was then fixed to the membrane in a vacuum oven at 80°C for 1 hour.

3.5.2.1.9 Preparation of Digoxigenin labelled DNA probes

PCR products were labelled with DIG using the DIG DNA labelling and detection kit (Boehringer Mannheim, Germany). A fragment of 96 bp (using P2 and P3) was used. The DNA was denatured by boiling for 10 minutes, incubated with 1 µl random hexanucleotide mix, 1µl dNTP labelling mix and 1 unit of Klenow enzyme for 2-16 hours at 37°C. This labelling reaction results in the random incorporation of DIG-11-dUTP (deoxy uracil triphosphate) every 20-25 nucleotides in the newly synthesized DNA (Feinberg and Volgenstein, 1983).

3.5.2.1.10 Hybridization of DIG labelled probes

Hybridizations were carried out as described by Boehringer Mannheim (Germany). Filters were prehybridized by incubating in hybridization solution

(5× SCC (sodium chloride and sodium citrate buffer), 5% (w/v) blocking solution, 0.1% (w/v) N-lauroylsarcosine Na-salt, 0.02% (w/v) SDS, 50%v/v formamide) for 1 hour at 42°C. Freshly denaturated probe was added (2-5µl) and hybridization was carried out for 2-16 hours at 42°C. Filters were washed twice in 2xSCC, 0.1%w/v SDS for 5 minutes at room temperature and then twice in 0.1xSCC, 0.1%w/v SDS for 15 minutes at 42°C.

3.5.2.1.11 Immunological detection of bound DIG labelled probes

All the incubations in this method were carried out at room temperature and, except for the colour reaction, with shaking.

Filters were washed briefly in Buffer 1 (100mM Tris, 150mM NaCl) and then blocked in Buffer 2 (Buffer 1 with 0.5% (w/v) blocking solution) for 30 minutes. After washing the filters were incubated in 10-20ml Buffer 1 containing 1:5000 (v/v) dilution of anti-DIG alkaline-phosphatase conjugate for 30 minutes. The unbound conjugate was removed by washing twice in Buffer 1 for 15 minutes. The filters were equilibrated in Buffer 3 (100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5) and a colour solution was added which consisted of 45µl nitroblue tetrazolium (NBT) solution (75mg /ml nitroblue tetrazolium salt in dimethylformamide), and 35µl X-phosphate (50mg/ml in 5-bromo-4-cloro-3-indolylphosphate, toludinium salt in dimethylformamide) in 20ml Buffer 3. The filters were then incubated in the dark for 1-24 hours until the colour developed and the reaction stopped by washing briefly in Tris-EDTA solution. Finally the filters were air dried.

3.6 Humoral immune response

3.6.1 Indirect fluorescent antibody test

An IFAT was used to quantify *T. gondii* specific antibodies in test sera from challenged sheep and cattle, based on that described by Munday and Corbould (1971).

3.6.1.1 Serum samples

Samples were collected as described in 3.4.1 Serum samples.

3.6.1.2 Antigen preparation

Whole killed *T. gondii* antigen using the S48 strain was prepared as described in 3.2.2 S48 strain.

3.6.1.3 Conjugate

Rabbit anti bovine IgG (whole molecule) FITC conjugate and pig anti sheep IgG (whole molecule) FITC conjugate were supplied by Sigma Chemical (UK).

3.6.1.4 Assay procedure

Five microlitres of a suspension of formalinised (0.2% v/v) tachyzoites (10^7per ml) were layered onto each well of 15 well HTC autoclavable slides (Cell-Line Ltd., USA) and left to dry overnight in a dust free area. The slides were fixed in methanol for ten minutes and then washed twice in PBS for 10 minutes each time. After washing, 5µl of diluted test sera and hyperimmune serum (diluted in PBS) as a positive control, were added to each well. The sera were tested at dilutions of 1:16,

1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, 1:4096, 1:8192, 1:16384, 1:32768 and 1:65536. One well contained 5µl of a non immune serum acting as a negative control and one well with PBS only as a background control for the FITC conjugated second antibody. The slides were incubated in a moist atmosphere at 37°C for 30 minutes and then washed in PBS for two ten minute periods.

All the wells were layered with 5µl of either rabbit anti bovine IgG (whole molecule) FITC conjugate (1/256 working dilution) or pig anti sheep IgG (whole molecule) FITC conjugate (1/40 working dilution) diluted in PBS containing 0.2% Evan's Blue. After incubation for another half an hour at 37°C in a moist atmosphere the slides were washed with PBS (3×10 minutes washes) and finally the slides were mounted using Citifluor (glycerol/PBS solution, Citifluor Ltd., UK) and viewed with UV light under an Olympus BX50 microscope using a U-MNB filter cube with a ×40 objective.

Values up to 1/32 were considered negative and values over 1/32 considered positive for ovine samples.

3.6.2 Enzyme-Linked Immuno-Sorbent assay

An ELISA to quantify IgG antibody to *T. gondii* on ovine and murine serum samples was used based on that described by Voller and collaborators (1976) and Buxton and co-workers (1988).

3.6.2.1 Serum samples

Samples from sheep and mice were collected as described in 3.4.1 Serum samples.

3.6.2.2 Antigen preparation

Water soluble fraction of *T. gondii* antigen using the RH strain was prepared as described in 3.3.1 Water soluble fraction of *T. gondii*.

3.6.2.3 Assay procedure

Dynatech 96 flat bottom plates (Dynatech Ltd., UK) were coated with 150µl of *T. gondii* antigen (estimated protein: 3µg/ml) diluted at 1:500 in 0.1M carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were then washed three times in PBS containing 0.05% Tween 20 (pH 7.4) (PBST).

Serial doubling dilutions (1:800 to 1:102400) of a standard high titre sheep antiserum to *T. gondii* were included, and 150 µl of test serum diluted 1:500 in PBS/1% BSA (Bovine Serum Albumin-99% albumin, BSA) (Sigma Chemical, UK) was added to the appropriate well and the plates incubated for 2 hours at 37°C in a moist atmosphere. After three washes with PBST, 150µl of conjugate (either pig IgG anti sheep IgG conjugated to horseradish peroxidase (1/500) or rabbit IgG anti mouse IgG conjugated to horseradish peroxidase (1/20000)) (Sigma Chemical, UK) diluted in PBST containing 1% BSA were added and incubated for a further 2 hours at 37°C as described above.

The plates were then washed three times with PBST and 150µl of ortho-phenylenediamine substrate (OPD, Sigma Chemical, UK) was added and incubated for 30 minutes in the dark at room temperature. The substrate consisted of 40mg OPD in 100ml phosphate/citrate buffer (0.02M citric acid, 0.05M sodium hydrogen phosphate, pH 5) plus 40µl H_2O_2. The enzyme-substrate reaction was stopped by the addition of 50µl of arrestor (2.5M H_2SO_4) to each well. The resulting colour changes were read on an ELISA plate reader measuring OD with a 492nm filter.

The OD of the standard high titre serum dilutions were used to construct a standard curve. The OD at a dilution of 1 in 500 was read and given the arbitrary value of 100% and all subsequent OD values within the experiment were expressed as a percentage of this with the following equation (Buxton *et al.*, 1988):

$$\frac{(\text{experimental OD}) - (\text{negative antigen OD})}{(\text{standard OD}) - (\text{negative antigen OD})} \times 100$$

A positive result was taken to be any reading above 25% for sheep and above 10% for the mice.

3.7 Cellular immune response

3.7.1 Antigen induced proliferation assay on peripheral blood mononuclear cells

A proliferation assay using PBM cells was set up to analyse the response of the cells to *T. gondii* antigen as described by Innes and collaborators (1995a).

3.7.1.1 PBMC preparation

PBM cells from challenge sheep and cattle were collected as described in 3.4.3.2 and resuspended at $4x10^6$cells/ml.

3.7.1.2 Antigen preparation

Water soluble extract of *T. gondii* was prepared as described in 3.3.1 Water soluble fraction of *T. gondii*.

3.7.1.3 Concanavalin A

Concanavalin A (Con A) (Sigma Chemical, UK), the lectin of the jack bean (*Canavalia ensiformis*), was used as positive control for the test, as it has been

described to selectively stimulate T lymphocytes (Janossy and Greaves, 1971; Rouse and Babiuk, 1974). In contrast untreated cells were used as a negative control.

3.7.1.4 Radiolabelling

All the cultures were pulsed with [^{3}H]-thymidine (The Radiochemical Centre, Amersham, Buckinghamshire, UK).

3.7.1.5 Assay procedure

Aliquots each of 100 µl of PBM cells and 100 µl of diluted *T. gondii* antigen were added to a 96 well round bottomed plate (Nunc, Denmark) in quadruplicate. Cells and antigen were cultured for five days at 37°C in a humidified 5% CO_2 incubator and pulsed for the final 18h with 0.5µCi (18.5 KBq)/ well of [^{3}H]-Thymidine, prior to harvesting onto filters. Cell associated radioactivity was quantified in a beta scintillation counter.

3.7.1.6 Determination of Stimulation index

The results reported as counts per minute (cpm) are the mean value of quadruplicate cultures. The differential incorporation of [^{3}H]-Thymidine between treated and untreated cultures was used as a measure of proliferation expressed as stimulation index (SI):

$$SI = \frac{\text{cpm of test culture}}{\text{cpm of negative control culture}}$$

3.7.2 Phenotypic analysis of peripheral blood mononuclear cells

An indirect immunofluorescence test was used to stain PBM cells from sheep and cattle as described by Innes and collaborators (1995a).

3.7.2.1 PBM cells preparation

PBM cells from challenge sheep and cattle were prepared as described in 3.4.3.3 Peripheral blood mononuclear cells for phenotypic analysis.

3.7.2.2 Monoclonal Antibodies

Appropriate monoclonal antibodies (MoAb) at a pre-determined optimal dilution were used to stain the different population of cells.

Table 3. 1 Monoclonal antibodies specific for distinct sheep and cattle leukocyte molecules

	Antibody specificity		
Species	**$CD2^+$**	**$CD4^+$**	**$CD8^+$**
sheep	36F	17D	7C2
cattle	CC42	CC8	CC63

The monoclonal antibodies specific for distinct sheep leukocyte molecules were obtained from the Basel Institute for Immunology (Switzerland) and the monoclonal antibodies specific for cattle leukocyte molecules were obtained from Institute for Animal Health (IAH) in Comptom (UK).

3.7.2.3 Conjugate

Fluoroscein isothiocyanate (FITC) conjugated anti-mouse immunoglobulin (Dakopatts, Denmark) was used.

3.7.2.4 Assay procedure

Aliquots containing 2×10^6 washed PBM cells were resuspended in 50µl of fluorescence-activated cell sorter (FACS) medium (HBSS supplemented with 2% FBS and 0.01M sodium azide) and mixed with 50µl of the appropriate MoAb at a predetermined optimal dilution. Cells and MoAbs were incubated at 4°C for 30 minutes and washed three times by repeated resuspension and centrifugation for 3 minutes at 100*g*. The supernatant fluid was removed and 50µl of a 1:50 dilution of a FITC conjugated anti-mouse was added to the cells. After a further 30 minute incubation the cells were washed as described above before fixing in PBS containing 1% paraformaldehyde.

3.7.2.5 Determination of stained cells

The percentage of stained cells was determined for each MoAb and a sample stained only with the FITC conjugate was used as a control. Ten thousand cells were analyzed per sample using flow cytometry (FACScan).

3.7.3 Cytokine analysis

Cell free peripheral blood plasma and supernatant from short-term cultured PBM cells were tested for the presence of IFN, either biologically active IFN using a bioassay described by Entrican and collaborators (1989, 1992) or IFNγ using an

enzyme immunoassay (EIA) test (CSL Veterinary, Australia) as described by the manufactures.

3.7.3.1 Peripheral blood plasma collection

Cell free peripheral blood plasma was collected as described in 3.4.2 Cell free peripheral blood plasma.

3.7.3.2 Supernatants from short term PBMC cultures

Supernatants from short term cultures of PBM cells were collected as described in 3.4.4 Cytokine analysis.

3.7.4 Bioassay for detection of biologically active IFN

This assay was performed as described by Entrican and co-workers (1989, 1992). Samples were tested for their ability to inhibit the replication of Semliki Forest virus (SFV) in ovine fibroblast cells.

3.7.4.1.1 Ovine fibroblast cells

ST-6 cells, cultured from an ovine adenocarcinoma of the small intestine by repeated passage *in vitro*, were used as the target cell in the assay. The derivation and characterization of these cells have already been described by Norval and co-workers (1981).

3.7.4.1.2 Semliki Forest virus

Semliki Forest virus (kindly provided by Dr. Entrican, MRI) was added to the cultures in order to measure the cytopathic effect (CPE) provoked.

3.7.4.1.3 Assay procedure

ST-6 cells were plated out in 96-well flat-bottomed microtitre plates (Costar, UK) at a density of 5×10^3 cell per well in 100µl of IMDM supplemented with 5% FBS. When the cells formed a confluent monolayer (3-4 days), neat test samples were added to quadruplicate well in 100µl volumes. ST-6 cells cultured with medium alone were used as a negative control and ST-6 cells cultured with SFV alone were used as positive control. Following a further 24 hour incubation the medium in the wells was removed and replaced with 100 tissue culture infective dose $(TCID)_{50}$ of SFV in 200µl of IMDM supplemented with 2% FBS. The plates were then incubated with the virus for 48 hours before the CPE was measured.

A sample was considered to be negative for interferon if the fibroblast cells have rounded up due to viral replication. Cells were protected from viral replication if biologically active IFN was present in the test samples. The assay was read visually and the % of CPE was estimated for each test sample.

3.7.4.2 Enzyme immunoassay for detection of IFNγ

This enzyme immunoassay was performed as described by manufacturers. This test consisted of a sandwich enzyme immunoassay able to detect IFNγ. This test is capable of detecting less than 50pg/ml of recombinant IFNγ, the approximate equivalent of less than 1 unit of IFNγ.

3.7.4.2.1 Assay procedure

Diluted samples were loaded onto microtitre plates coated with antibody to IFNγ and incubated at room temperature ($22^\circ \pm 5^\circ$C for 60 ± 5 minutes). Unbound material was then removed by washing (six times) with wash buffer. After the sixth wash the plates were placed face down on clean filter paper and allowed to drain in order to remove as much remaining wash buffer as possible. Then freshly prepared conjugate, comprising horseradish peroxidase labelled anti-bovine IFNγ, was added

to react with IFNγ bound to the antibody coated to the solid support, incubated as above and unreacted conjugate was removed by washing as previously described. Enzyme substrate containing H_2O_2 was added and incubated for 30 minutes at room temperature. The reaction was finished when enzyme stopping solution with 0.5M H_2SO_4 was added.

The rate of conversion of substrate is proportional to the amount of bound IFNγ. The amount of colour development was estimated spectrophotometrically.

3.8 Parasite detection in tissue samples

To determine the presence of *T. gondii* within the tissues of the challenged animals, half of the animals in each group were killed at six weeks and at six months after challenge and post-mortem examination was carried out. Samples of the several tissues were collected in order to be analyzed for the presence of *T. gondii* (see Chapter 4 and Chapter 5 for further details).

3.8.1 Histopathology examination

3.8.1.1 Samples collected

Tissue samples were taken from animals necropsied either six weeks or six months after infection for microscopic examination. Samples of the following tissues were collected: brain, spinal cord, cardiac muscle, skeletal muscles (psoas, gracillis), liver, mesenteric lymph node, lung and kidney immediately fixed in 10% formal saline and processed for microscopically examination.

3.8.1.2 Tissue section preparation

Tissues were fixed in 10% formal saline (40% formaldehyde and 0.9% sodium chloride) for 2 to 4 hours, then post-fixed for 1h using modified Bouin

(saturated aqueous picric acid 1%, 40% formaldehyde and glacial acetic acid). The tissues were processed routinely in a Hypercenter-XP (Shandon, UK) and embedded in paraffin wax. Sections 4μm thick were cut, placed on poly-l-lysine coated slides, and dried overnight at 37°C.

3.8.1.3 Haematoxylin and eosin stain

Sections were dewaxed in xylene, rehydrated by a graded series of alcohol and washed in tap water. They were stained in Mayers haematoxylin for 5 minutes, differentiated by 1% HCl in 70% alcohol and blued up in Scott's Tap Water Substitute. A counterstain of eosin was used before dehydration through a graded series of alcohol into xylene. The slides were mounted permanently using CoverBond-Mounting Media (xylene based mountant). Nuclei were stained blue and the cell cytoplasm was stained pink.

3.8.2 Bioassay in mice

To define the presence of viable *T. gondii,* tissue samples of brain, cardiac muscle and skeletal muscle (psoas and gracillis) were collected from the challenged sheep and cattle, processed and intraperitoneally inoculated into three mice.

3.8.2.1 Sample preparation

Approximately 1 to 3 gr. of infected tissue was removed and placed in a sterile container. The sample was homogenized with 10ml of 0.25% trypsin in PBS and incubated with gently agitation for 60 minutes at 37°C. The large particles were removed by filtering with fine mesh gauze and then pelleted at 1000*g* for 15 minutes.

3.8.2.2 Assay procedure

Once the samples were processed, three female Swiss White mice were each inoculated with 0.5ml of the processed sample by i.p. injection. The animals were inspected daily for signs of a febrile response that may indicate acute toxoplasmosis. Mice which showed signs of illness (tottering gait, hunched appearance alongside evidence of emaciation and dehydration) were culled immediately and a sample of peritoneal exudate removed and inspected for tachyzoites by microscopic examination. Mice which survived were bled at 8 weeks post inoculation and the presence of specific *T. gondii* antibodies detected by IgG ELISA as described on 3.6.2 Enzyme-Linked Immuno-Sorbent assay.

A group of mice from a *Toxoplasma* free colony which received no inoculum was used as negative controls.

3.8.3 Detection of *T. gondii* DNA by the B1 polymerase chain reaction

A B1 PCR technique was used to detect and amplify specific *T. gondii* DNA from tissues of orally challenged sheep and cattle.

3.8.3.1 Samples collected

Tissues were taken from animals necropsied either six weeks or six months after challenge. Tissue samples of brain, cardiac muscle and skeletal muscles (psoas, gracillis), were removed, placed in a sterile container and stored at -20°C until required.

3.8.3.2 DNA extraction from tissue samples

Each tissue was finely chopped and processed as described in 3.5.2.1.2 DNA extraction from peripheral blood mononuclear cells.

3.8.3.3 S48 T. gondii *preparation and DNA extraction*

DNA was extracted from S48 tachyzoites as described on 3.5.2.1.3 Preparation *of T.* gondii *tachyzoites of* the S48 strain and 3.5.2.1.4 *T. gondii* DNA *extraction.*

3.8.3.4 Quantification of DNA from T. gondii

The concentration of DNA was assessed by taking readings of Optical density as described in 3.5.2.1.5 Quantification of *T. gondii* DNA.

3.8.3.5 The B1 PCR

The nested B1 PCR was carried out in a Hybaid thermal cycler over 25 cycles in 50µl volumes as described in 3.5.2.1.6 B1 polymerase chain reaction.

3.9 Statistical analysis

A Student's t-test was calculated, using the mean values in each group to determine significant differences, and the data were tested at a $P<0.05$, $P<0.01$ significant level.

Chapter 4

Oral infection of sheep with a titrated dose of *Toxoplasma gondii* oocysts

Aims

To examine parasitaemia and host humoral immune response in sheep given a titrated dose of *T. gondii* oocysts of the M3 isolate.

To establish the dose of oocysts required to produce a detectable amount of *T. gondii* parasites within sheep tissues.

4. Oral infection of sheep with a titrated dose of *T. gondii* oocysts

4.1 Introduction

In the early nineteen seventies the identification of the cat as the definitive host of *T. gondii* occurred with the discovery of oocysts in its faeces (Hutchison *et al.*, 1969, 1970, 1971; Work and Hutchison, 1969a, b; Frenkel *et al.*, 1970; Dubey *et al.*, 1970a, b; Sheffield and Melton, 1970; Jackson and Hutchison, 1989).

Transmission of infection by ingestion of *T. gondii* oocysts is the main cause of infection in herbivores, along with congenital transmission. This was confirmed in several reports on natural outbreaks of clinical toxoplasmosis in sheep where the source of infection was *T. gondii* oocysts (Plant *et al.*, 1974; Dubey *et al.*, 1986; Faull *et al.*, 1986).

Infection with *T. gondii* usually results in the formation of parasite cysts in the host tissues, characterizing subclinical chronic infection (Frenkel *et al.*, 1970). In the majority of intermediate hosts, such as sheep, this parasite stage may persist for the life time of the host (Lainson, 1959; Jacobs *et al.*, 1960a, b; Remington and Cavanaugh, 1965; Robertson, 1966; Conley and Jenkins, 1981; Dubey and Beattie, 1988).

The first study of experimentally induced toxoplasmosis in sheep orally infected with 10^4 oocysts was reported in 1975 by Beverley and collaborators. The objective of this study was to investigate the possibility of oocyst formation in sheep as well as the development of *T. gondii* infection in sheep following oral infection with oocysts. No evidence was found that *Toxoplasma* oocyst formation occurs in sheep, and it was stated that a primary infection with *T. gondii* oocysts in pregnant sheep can be just as harmful to the foetus as an infection using *T. gondii* tissue cysts

(Beverley *et al.*, 1975). Since then, several research groups have used *T. gondii* oocysts to conduct experimental infections of sheep. Dubey and Sharma (1980) showed that after oral infection of naive 4- to 6-year-old sheep, with either 10^2 or 10^5 oocysts of the GT-1 strain, there was a transient and low parasitaemia. *Toxoplasma* was isolated from numerous tissues of orally infected animals by mouse inoculation. It was stated that the persistence of the parasite in skeletal muscle of sheep indicates that this may be a favoured site of *T. gondii* in sheep and the difference in the doses administered did not affect the distribution of the parasite in the tissues (Dubey and Sharma, 1980).

In 1988, McColgan *et al.*, carried out a titration experiment in which fifty-nine ewes, seronegative to *T. gondii*, were allocated into four groups which received 20, 2×10^2 or 2×10^3 M1 *Toxoplasma* oocysts. It was demonstrated that 2×10^2 oocysts given orally to sheep was the threshold infective dose, defined by febrile response and seroconversion. During this experiment, sheep given 2×10^3 M1 oocysts developed a significant febrile response and seroconverted with significantly higher %OD by IgG ELISA than the animals which received the lower doses.

Garnham and Lainson (1960) first reported the persistence of *T. gondii* cysts in ovine tissues after parenteral inoculation of sheep with tissue cysts. The infected animals were killed at either 112 or 150 days after infection and the parasite was isolated from brain, skeletal muscle, heart, liver and kidney by intraperitoneal inoculation of 1ml of the tissue suspension into pairs of multimammate rats (*Mastomys coucha*).

The aims of the present study were to describe the parasitaemia and humoral response in sheep experimentally infected with a titrated dose of *T. gondii* oocysts and to ascertain the dose of oocysts which would produce detectable levels of parasite in sheep tissues.

4.2 Materials and methods

4.2.1 Animals

Twelve 9-month-old male Greyface sheep shown to be seronegative to *T. gondii* by ELISA were used in this study.

Mature Swiss White mice of both sexes from *Toxoplasma* free colonies were used to passage and maintain the S48 strain of *T. gondii* tachyzoites as described in Chapter 3 (3.1.3, 3.2).

Cats of both sexes, under six months of age and seronegative for antibodies to *T. gondii*, obtained from a *Toxoplasma* free colony in the University of Newcastle (UK) were used to produce M3 isolate *T. gondii* tachyzoites (Chapter 3, 3.1.4).

4.2.2 Toxoplasma gondii

To produce sporulated oocysts of the M3 isolate of *T. gondii,* cats were fed a suspension of approximately 10^3 tissue cysts mixed in a small amount of food and their faeces were collected daily and analyzed for the presence of oocysts (Chapter 3, 3.2.1.3).

Toxoplasma gondii tachyzoites of the S48 strain were prepared as described in Chapter 3 (3.2.2), and used as antigen in the serological analysis. Tachyzoites were counted using a haemocytometer and preparations with less than 2% cellular contamination were used as antigen for the IFAT (Chapter 3, 3.3.2).

4.2.3 Experimental design

Sheep were allocated into three groups as described in Table 4. 1. On day 0 suspensions containing 10^3, 10^4 and 10^5 sporulated *T. gondii* oocysts of M3 isolate were administrated to the sheep by gavage (Table 4. 1).

Table 4. 1 Experimental design

Group	Species	Animal numbers	M3 Oocysts dose
1	sheep	S1, S2, S3, S4	10^5
2	sheep	S5, S6, S7, S8	10^4
3	sheep	S9, S10, S11, S12	10^3

s: sheep

4.2.4 Clinical analysis

Rectal temperatures were recorded daily from 3 days before and until 14 days after oral infection with a clinical electronic thermometer.

Blood samples were taken daily from the jugular vein from 3 days before and until 14 days after infection, PBM cells were separated and examined using the B1 PCR to detect any specific *T. gondii* DNA (Chapter 3, 3.4.3).

4.2.5 Serology

Blood samples were collected from the jugular vein of sheep into preservative free evacuated blood collection tubes and allowed to clot. Following retraction of the

clot and centrifugation at 500*g* for 15 minutes, serum was removed, and stored at -20°C until required. Serum samples were collected every second day from 3 days before and until 14 days after infection to examine for the presence of specific antibodies (see Appendix Chapter 4 for further details).

An IFAT was used to quantify *T. gondii* specific antibodies in test sera, based on the method described by Munday and Corbould, 1971 (Chapter 3, 3.6.1).

An ELISA was used to detect IgG antibody to *T. gondii.* The method was described by Voller and collaborators (1976) and Buxton and co-workers (1988) (Chapter 3, 3.6.2).

4.2.6 Parasite detection

All the animals were killed 6 weeks after infection.

4.2.6.1 Histopathology

Samples of brain, cardiac muscle, skeletal muscle (gracillis and psoas), lung, liver, kidney and mesenteric lymph node were removed and immediately fixed in 10% formal saline, then processed using the method described in Chapter 3 (3.8.1). Sections 4μm thick were cut from the paraffin wax embedded tissues, placed on poly-l-lysine coated slides, and stained following the method outlined in Chapter 3 (3.8.1.3) prior to microscopic examination.

4.2.6.2 Polymerase chain reaction

Samples of brain, cardiac muscle, and skeletal muscle (gracillis and psoas) were taken for specific DNA detection using the B1 PCR. To address the problem of sampling error four large samples of approximately 100g were taken from different sections of each organ and stored at -20°C prior to extraction of DNA for PCR analysis. Approximately two grams of each sample were analysed and when a

positive result was obtained the remainder of the sample was not tested. The PCR product was confirmed to be *T. gondii* DNA by Southern blotting and immunological detection of bound DIG labelled probes to *T. gondii* DNA described in Chapter 3 (3.5.2).

4.3 Results

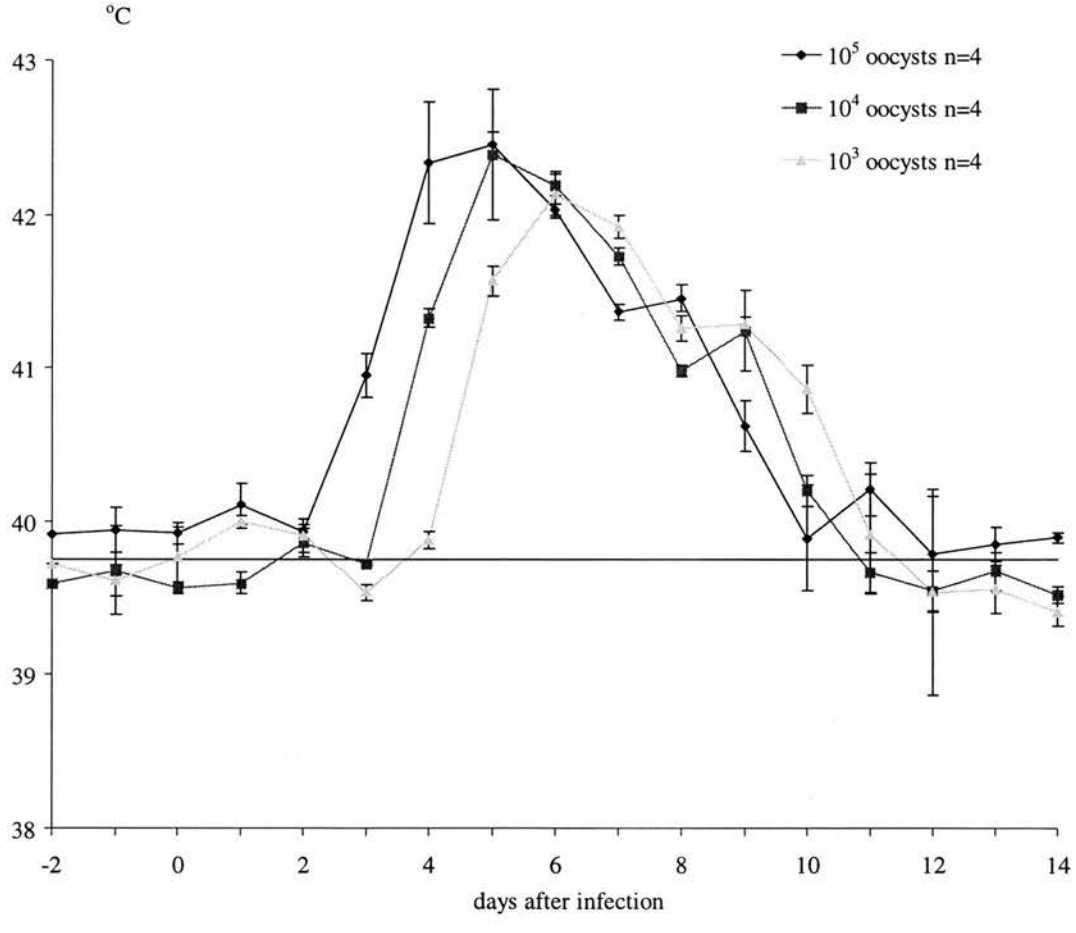
4.3.1 Clinical response

4.3.1.1 Temperature response

The rectal temperature data were analysed by taking the mean of the temperature values for all the animals, recorded before infection on day 0, as a base line value. A dose dependent febrile response was observed when rectal temperatures were recorded daily for 14 days following infection. All three groups showed rectal temperatures below 40°C before infection. The febrile response, which reached values of greater than 42°C, lasted for five days ($P<0.01$) (Figure 4.1). Details of the individual animals are displayed in Appendix Chapter 4 (Table 2 to Table 4).

Group 1 (10^5 oocysts) showed a significant response ($P<0.01$ Student's t-test) compared to base line value on day 4 after infection which reached a peak on day 5 before starting the return to base line value by day 8. However, the other two groups (Group 2: 10^4 oocysts and Group 3: 10^3 oocysts) had a significantly later response ($P<0.01$ Student's t-test) compared to group 1, starting the return to base line values on day 9 post infection. Group 2 reached the peak temperature on day 5 and group 3 peaked on day 6 after infection.

Figure 4. 1 Mean (±se) rectal temperatures of sheep following primary oral infection with *T. gondii* oocysts of the M3 isolate



The mean base line value is represented by a continuous line at 39.75°C. se: standard error

4.3.1.2 Parasitaemia

The results obtained from the analysis of PBM cells for the presence of *T. gondii* are illustrated in Table 4. 2. Using the B1 PCR *T. gondii* DNA was not detected in any of the sheep before infection on day 0. *T. gondii* specific DNA was detected and amplified in the PBM cells of each of the four animals infected with 10^5 oocysts. No *T. gondii* was detected in any of the animals from Group 2 and only one animal from Group 3 gave a positive result on day 9 after infection. These results show that the high-dose animals had a greater parasitaemia than the medium and low-dose.

Table 4. 2 Detection of *T. gondii* DNA in the PBM cells of sheep orally infected with 10^5, 10^4 and 10^3 *Toxoplasma* oocysts of the M3 isolate

Days after infection	Group 1				Group 2				Group 3			
	10^5 oocysts				10^4 oocysts				10^3 oocysts			
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
0	-	-	-	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	-	-
2	✤	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	✤	✤	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	✤	✤	-	-	-	-	-	-	-	-	-
7	-	-	-	✤	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-
9	✤	-	-	-	-	-	-	-	-	✤	-	-
10	-	✤	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-

✤: detection and amplification of *T. gondii* DNA in PBM cells by the B1 PCR

-: no detection of *T. gondii* DNA in PBM cells by the B1 PCR

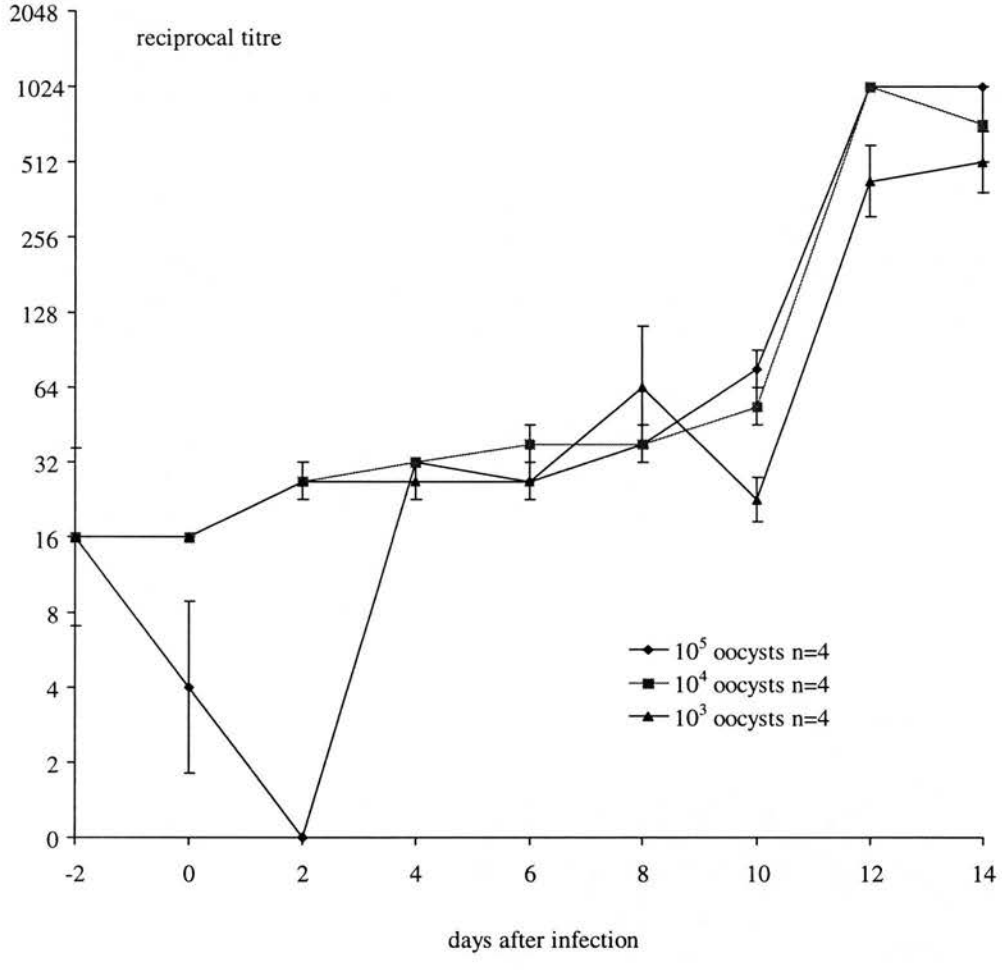
4.3.2 Serology

4.3.2.1 Indirect fluorescent antibody test

All the animals in this experiment manifested specific seroconversion after oral infection with 10^5, 10^4 or 10^3 *T. gondii* oocysts of the M3 isolate using the IFAT to detect *T. gondii* specific IgG. Serum samples from these animals were analysed using doubling dilutions starting from 1:16. The results are illustrated in Figure 4. 2. Details of individual animals are shown in Appendix Chapter 4 (Table 5 to Table 7).

Animals infected with either 10^5 or 10^4 oocysts show a 6-fold increase in specific antibody titre from base line values on day 12 after infection. Animals given the lowest dose (10^3 oocysts) show a 5 -fold increase from base line values on day 12 after infection.

Figure 4. 2 Mean (±se) titre of IgG antibody in sheep orally infected with *T. gondii* oocyst of the M3 isolate measured by IFAT



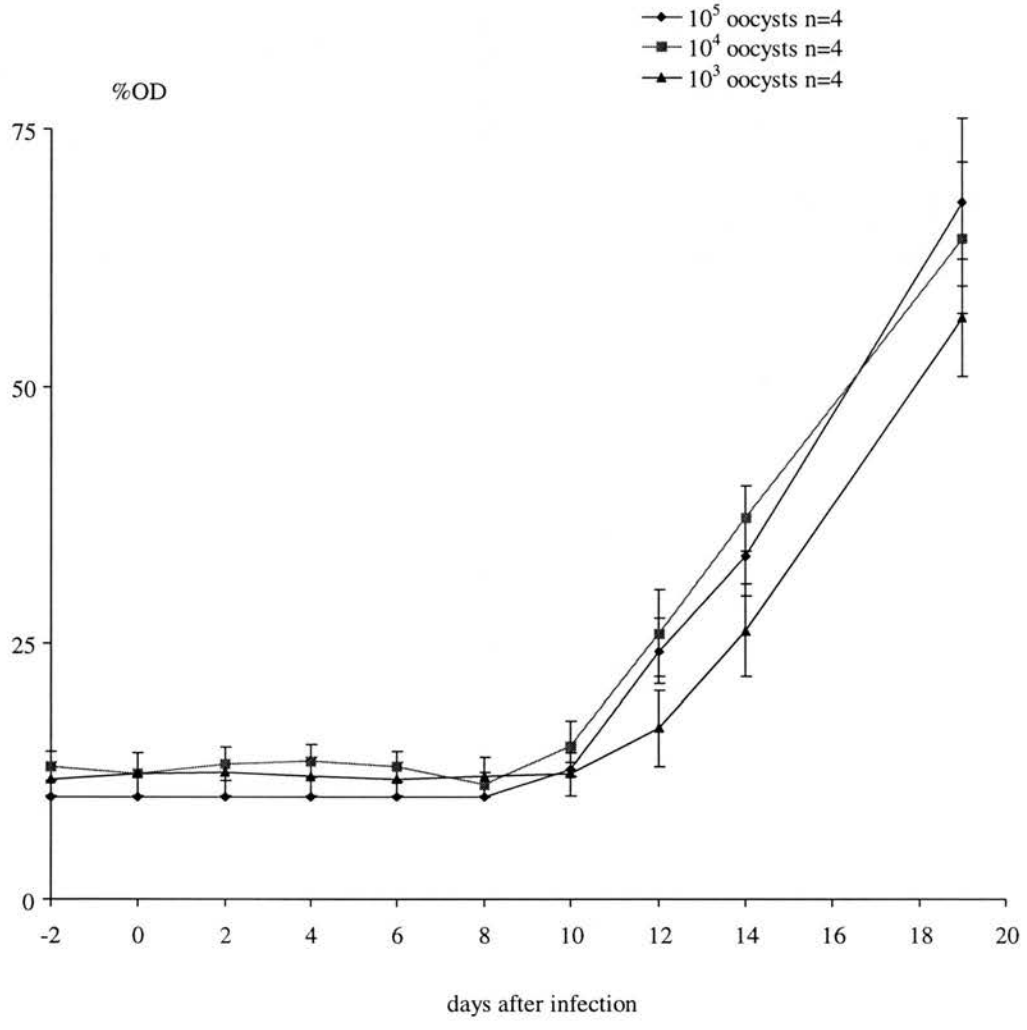
se: standard error

4.3.2.2 Enzyme-linked immuno-sorbent assay

The results from the analysis of serum samples using the IgG ELISA are illustrated in Figure 4. 3. The data were expressed as a percentage of a standard high titre positive serum. Experience has shown that adult ewes with a 25 % or less OD using the ELISA are seronegative (Buxton *et al.,* 1991) (Chapter 3, 3.6.2). Seroconversion was demonstrated in all the animals when the serum samples were analysed by ELISA for the presence of *T. gondii* specific IgG. Details of individual animals are shown in Appendix Chapter 4 (Tables 8 to 10).

Group 1 (10^5 oocysts) had a mean % OD greater than 25 on day 14 after infection while group 2 (10^4 oocysts) did so on day 12, although a statistically significant increase in the %OD was not detected in both groups until day 14 after infection ($p<0.01$ Student's t-test). Although in group 3 (10^3 oocysts) a % OD just greater than 25 was recorded on day 14 after infection, there was no statistically significant increase in the level of IgG antibodies until day 19 after infection.

Figure 4. 3 Mean (±se) % of OD IgG antibody in sheep orally infected with *T. gondii* oocyst of the M3 isolate measured by ELISA



The data were expressed as a percentage of a standard high titre positive serum. An OD below 25 % is considered seronegative (Buxton *et al.*, 1991).

se: standard error

4.3.3 Parasite detection

4.3.3.1 Histopathology

No tissue cysts or histopathological changes were observed in any of the 12 animals after examination of tissues (brain, cardiac muscle, skeletal muscle (gracillis and psoas), lung, liver, kidney and mesenteric lymph node) stained with H&E.

4.3.3.2 PCR

The result from the analysis of tissue samples using the B1 PCR are illustrated in Table 4. 3. Specific DNA was detected in 8 of the 12 sheep. It was more readily detected in samples from group 1 (10^5 oocysts) than from groups 2 (10^4 oocysts) and 3 (10^3 oocysts) and was mainly detected in brain and heart. Only one sample of gracillis muscle from the sheep in group 1 (10^5 oocysts) gave a positive reaction.

Table 4. 3 Detection of *T. gondii* DNA in tissues of sheep orally infected with 10^5, 10^4 or 10^3 *Toxoplasma* oocysts of M3 isolate by the B1 PCR

Tissue samples	Group 1				Group 2				Group 3			
	10^5 oocyst				10^4 oocysts				10^3 oocysts			
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
brain	✤	✤	-	✤	-	-	✤	✤	-	-	-	-
cardiac muscle	✤	✤	-	✤	✤	-	-	-	-	✤	✤	-
skeletal muscle psoas	-	-	-	-	-	-	-	-	-	-	-	-
skeletal muscle gracillis	-	-	-	✤	-	-	-	-	-	-	-	-

✤: detection and amplification of *T. gondii* DNA in PBM cells by the B1 PCR

-: no detection of *T. gondii* DNA in PBM cells by the B1 PCR

4.4 Discussion

The M3 isolate of *T. gondii* used in this study is a complete isolate, tissue cysts from infected mice result in oocyst production if ingested by seronegative cats (Chapter 3, 3.2.1).

The study demonstrates that infection of sheep with the M3 isolate results in parasites being present in the tissues, but detection appears dependent on the number of oocysts ingested by the sheep. Parasites were more consistently detected in the group of sheep given 10^5 oocysts compared to 10^3, brain and cardiac muscle being the best candidate tissues for detection.

There are conflicting reports in the literature concerning the favoured tissue for detection of *T. gondii* parasites. Various workers have reported the presence of *T. gondii* in tissues following experimental infection of sheep using several infection routes and different parasite stages (Jacobs *et al.,* 1963; Jacobs and Hartley 1964; Dubey and Sharma 1980; Dubey, 1984). When Dubey and Sharma (1980) orally dosed seronegative ewes with 10^5 sporulated *T. gondii* oocysts of the GT-1 strain, they were consistently able to isolate of the parasite from skeletal muscle of sheep killed between day 7 and 119 after infection, indicating that this may be a favoured site of *T. gondii* persistence in sheep. A similar result was reported by Jacobs and collaborators (1963) where they also detected *T. gondii* more frequently in muscle. However, the results from this study would suggest that the brain and heart are the favoured site for detection of *T. gondii* and indicate that a 10-fold and a 100-fold difference in oocyst dose affects the frequency of detection of *T. gondii* in the tissues. These results agree with those reported by Dubey and Sharma (1980) where a 1,000-fold difference in oocyst dose from 10^2 to 10^5 influenced the frequency of detection of parasite cysts in the tissues from infected sheep. In the study reported by Dubey and Sharma (1980) only one animal was killed at each time point and therefore it is difficult to draw definitive conclusions from their study.

While in the study reported here the sheep were killed 6 weeks after infection, and Garnham and Lainson (1960) reported the presence of parasite for at least 9 months after infection, it is generally assumed that sheep infected with a complete isolate of *T. gondii* will remain persistently infected for life (Uggla and Buxton, 1990). However, the size of the tissue sample examined is crucial to detection, and a negative result from any sample does not necessarily mean that the whole tissue is free of parasite. The problem of sample error when working with large animal species can only be avoided if the whole animal is examined, which is clearly impractical.

There are various techniques available to detect *T. gondii* within tissues. Methods for detecting or isolating *T. gondii* in body fluids or tissues offer a more specific means of documenting active infection than serum antibody titre. In this study *T. gondii* parasites were not observed on hematoxylin and eosin tissue sections from the infected sheep. This may have been due to a combination of low density of parasites within the tissue and the inevitable sample error. Also the sheep were killed at 6 weeks after infection and therefore any *T. gondii* cyst present in the tissues would be expected to be relatively immature. The detection of *Toxoplasma* parasites in tissue sections of infected sheep by histology may prove difficult due to the morphological similarities between *T. gondii*, *N. caninum* and *Sarcocystis spp.* Therefore more specific tests are required to show the presence of *T. gondii*. Isolation of *Toxoplasma* is usually carried out by inoculation of samples into susceptible laboratory animals. Mice are most commonly used, since *T. gondii* is highly infective to mice (Dubey *et al.*, 1988b; 1989). Depending on the virulence of the strain, mice develop either an acute infection with parasite-rich ascitic fluid or a chronic infection characterized by the presence of *T. gondii* cysts in the brain (Derouin *et al.*, 1987). PCR test has been used to detect *T. gondii* specific DNA in a wide selection of body fluids from several animal species: ocular fluid in horses (Turner and Savva, 1991), lymph and blood from experimentally infected sheep (Wastling *et al.*, 1993; Buxton *et al.*, 1994), placenta and foetal tissues from ovine abortions (Steuber *et al.*, 1995) and aqueous humor, cerebrospinal fluid, serum and blood samples from infected cats and dogs (Stiles *et al.*, 1996). Since a study reported by Wastling *et al.* (1993)

elegantly demonstrated the very close agreement between the PCR technique and mouse inoculation to detect parasitaemia in the same sample (Wastling *et al.*, 1993), the B1 PCR was used to analyse tissue samples in this study in preference to the mouse inoculation assay. The B1 gene is reported to be highly conserved across strains of *T. gondii* and has a high copy number in the *T. gondii* genome (Burg *et al.*, 1989). While the PCR test is extremely sensitive it only proves the presence of specific *T. gondii* DNA within the samples; unlike the mouse inoculation technique, PCR does not distinguish between live and dead parasites.

A febrile response around 4 to 6 days following infection of sheep with *T. gondii* is a very consistent clinical finding (Smith, 1961; Spence *et al.*, 1978; Miller *et al.*, 1982; Dubey, 1984; McColgan *et al.*, 1988; Buxton *et al.* 1991, 1994). Similar results to those obtained in the study reported here with group 3 (10^3 oocysts) were reported by Buxton and collaborators (1991) when they orally infected 14-month-old Scottish blackface ewe-lambs with $2x10^3$ sporulated oocysts of the same isolate (M3) with onset of pyrexia on day 5 after infection, and lasting for 5 days.

In 1984, Dubey published results recorded following oral infection of lambs, rams and ewes with 10^4 infective oocysts of the GT-1 strain of *T. gondii*. He showed that all sheep became febrile (greater than 40°C) between 3 and 7 days after infection and the fever lasted 2 to 7 days. When the same dose was used in the present experiment, statistically significant febrile response was not recorded until day 5 in any of the animals from group 2 (10^4 oocysts). This may reflect the different characteristics of the *T. gondii* strains used in each study. In the study reported here the onset and duration of the febrile response appeared to correlate with the infective dose. The highest dose (10^5 oocysts) induced the earliest and largest duration of temperature response in the sheep. A similar effect of oocyst dose and resultant temperature response in sheep was not observed by Buxton *et al.* (1988). However, in their study a different *T. gondii* isolate was used, but perhaps more importantly the dose range was narrower (2×10^3 to 12×10^3 oocysts) compared to the study reported here (10^3 to 10^5 oocysts).

In 1909, Nicolle and Manceaux discovered *T. gondii* by microscopic examination of a blood smear from an African rodent. However, direct observation of this parasite in blood is rare, and parasitaemia can usually be only demonstrated by more sensitive techniques which include inoculation of blood into mice, tissue culture or use of the PCR technique. Previous studies examining the presence of *T. gondii* in blood have demonstrated the sensitivity of the PCR technique (Johnson *et al.*, 1990; Ho-Yen *et al.*, 1992; Filice *et al.*, 1993; Dupoy-Camet *et al.*, 1993; Wastling *et al.*, 1993; Paugam *et al.*, 1995).

Detection of parasitaemia following experimental infection of sheep appears to correlate with the dose of oocysts used. In this study presence of parasite in blood was more frequently detected when 10^5 oocysts were used to infect sheep. These results correlated well with those reported by Dubey and Sharma (1980) who also reported that detection of parasitaemia was dependent on the dose of oocysts used to infect the sheep. In the study reported here parasitaemia was detected using PCR technique whereas Dubey and Sharma (1980) used mouse inoculation.

A common feature of *T. gondii* infection is the development of specific antibodies in the host (Dubey and Beattie, 1988). Detection of specific *T. gondii* antibodies is frequently used as a diagnostic aid (Uggla, 1986). In the study reported here the magnitude of the antibody response appeared to correlate with the dose of oocysts used to infect the sheep. Similar responses were observed in sheep infected with 10^5 and 10^4 but significantly less antibody was detected in the sheep infected with 10^3 oocysts and the onset of seroconversion was significantly later. These results corroborate those reported by McColgan and collaborators, (1988) where they describe a similar correlation between oocyst dose and onset of specific seroconversion.

The onset of seroconversion appeared to vary depending on the serological test used. The IFAT showed a significant increase in specific *T. gondii* antibodies on day 12 after infection, whereas this occurred on day 14 after infection using the ELISA. The discrepancy between the two tests may reflect differences in the antigens used to detect the antibodies. In the case of the IFAT whole fixed tachyzoites are

used whereas the antigen in the ELISA is a crude water soluble fraction of tachyzoites.

4.5 Conclusions

In conclusion, the results described here demonstrate that 10^3, 10^4 and 10^5 *T. gondii* oocysts of the M3 isolate are capable of inducing symptoms of infection, including pyrexia, seroconversion and detection of *T. gondii* in blood and tissues.

The sheep receiving the highest dose of oocysts showed the earliest onset of pyrexia, parasites were detected most frequently in blood and tissues and showed an earlier onset and greater magnitude of specific antibody response compared to the lowest dose group (10^3 oocysts).

Infection of sheep with 10^5 oocysts consistently produced a detectable level of *T. gondii* within tissues and therefore may be a useful dose to conduct the comparative experiment between sheep and cattle described in the following chapters.

Chapter 5

Detection of *T. gondii* in sheep and cattle following oral infection

Aims

To examine the presence of *T. gondii* in various tissues taken from sheep and cattle at six weeks and six months following oral infection with 10^3 or 10^5 sporulated oocysts

5. Detection of *T. gondii* in sheep and cattle following oral infection.

5.1 Introduction

Toxoplasmosis was first described in humans in 1923 in the retina of a hydrocephalic child by Janku (cited Dubey and Beattie, 1988), although a number of organisms reported earlier were later identified as *Toxoplasma gondii* (Kean, 1972). Pikerton and Weinman (1940) reported the first case of the disease in a patient who suffered a generalized and fatal illness with enlarged lymph nodes and areas of necrosis in many organs. However it was not until 1954 that the consumption of infected undercooked meat was recognised as a major source of infection for people (Weinman and Chandeller, 1954). Years later *T. gondii* bradyzoites within tissue cysts were characterized as the infective agents (Jacobs *et al.*, 1960a).

Infection with *T. gondii* is the most common parasitic infection world-wide with an estimated prevalence of 1 to 2 billion people (Chang, 1996). Transmission to people occurs following ingestion of sporulated oocysts, or bradyzoites within the cysts, contained in the tissues of numerous food animals. The frequency of infection is extremely variable in the different regions of the world. The presence of feline population is considered an important factor influencing the frequency of *Toxoplasma* infection as was demonstrated by Wallace (1969) when he found human infection prevalent on Pacific atolls on which there were cats, but virtually absent where there were none. Seroprevalence in the human population ranges from 0 to 90% (reviewed by Dubey and Beattie, 1988) and infection is more common in warm climates and in low-lying areas than in cold climates and mountainous regions, where conditions for sporulation and survival of oocysts are less favourable. The

prevalence of *T. gondii* infection varies between ethnic groups, but this is due to sanitary and cooking habits rather than to genetic differences. A seroprevalence of 80% has been reported from Paris where undercooked meat is often eaten (Desmonts, 1961). However, lower seroprevalences (10-40%) have been reported in countries from South-East Asia where meat is cooked thoroughly (Jacobs *et al.*, 1960b; Zuber and Jaquier, 1995). There is evidence supporting the hypothesis that cattle are not favoured host for *T. gondii* and that in general infection is more likely to occur from consumption of pork, lamb and goat's meat than beef (Dubey, 1980; Dubey, 1986a, b; Dubey and Towle, 1986, Dubey and Beattie, 1988). Consumption of cured pork has been recently recognised as an important source of *T. gondii* infection (Buffolano *et al.*, 1996).

The presence of *T. gondii* cysts in the tissues of naturally and experimentally infected sheep have been reported (Jacobs *et al.*, 1960a, b; Jacobs *et al.*, 1963; Jacobs and Hartley, 1964; Dubey and Sharma, 1980). *Toxoplasma gondii* has been detected from soft tissues such as brain, cardiac muscle, skeletal muscle, small intestine, liver and diaphragm. The brain is the most frequently infected tissue in either aborted foetuses or live animals born to infected ewes and has been suggested as the organ of choice for isolating *T. gondii* for diagnostic purposes (Uggla *et al.*, 1987).

In contrast, the parasite has rarely been isolated from naturally infected bovine tissues (reviewed by Dubey, 1986b).

Little is known of the density of *T. gondii* tissue cysts in organs of food animals. It has been estimated that the number of *T. gondii* tissue cysts per gram of tissue of food animals such as pigs may be low (less than 1 tissue cyst/50gr). As a consequence, it is generally not practical to demonstrate the presence of the parasite by histological examination of tissues (Dubey *et al.*, 1996). Therefore a concentration bioassay technique is often used to demonstrate *T. gondii* in tissues of food animals. The most specific, but least

practical, method of isolating the parasite from infected animals is by feeding tissues to seronegative cats and subsequently demonstrating oocysts in the faeces several days later (Dubey and Frenkel, 1976). Alternatively, viable parasites can be detected in infected animals by inoculating mice with tissue samples as they are extremely vulnerable to *T. gondii* infection (Araujo *et al.*, 1976; Williams *et al.*, 1978).

Several studies have shown the sensitivity of the PCR for detection of *Toxoplasma* DNA in various biological samples (Johnson *et al.*, 1990; Wheeler *et al.* 1990; Turner and Savva, 1991; Ho-Yen *et al.*, 1992; Filice *et al.*, 1993,; Dupoy-Camet *et al.*, 1993; Wastling *et al.*, 1993). In addition, studies have compared the traditional methods of *Toxoplasma* diagnosis, which include detection of specific antibodies, mouse inoculation and immunohistochemistry, with the PCR and have shown a good correlation between the different techniques in the diagnosis of toxoplasmosis (Greig *et al.*, 1993; Steuber *et al.*, 1995; Wastling *et al.*, 1993). There is also a very good correlation between PCR and mouse inoculation in the detection of parasitaemia (Wastling *et al.*, 1993).

Results from the previous study (reported in Chapter 4 of this thesis) showed that the M3 isolate of *T. gondii* is able to cause infection of ovine tissues and that 10^5 sporulated oocysts consistently produced a measurable amount of *T. gondii* within the tissues of infected sheep.

The main objective of this study was to compare the presence of *T. gondii* within tissues of sheep and cattle following oral infection with sporulated oocysts. The parameters examined were whether oocyst dose affected the frequency of *T. gondii* detection within tissues, the distribution of *T. gondii* within ovine and bovine tissues and whether there was difference in tissue distribution and frequency of parasite detection in tissues collected six weeks or six months following infection.

5.2 Materials and Methods

5.2.1 Animals

Ten adult sheep (ages ranging from 2 to 5 years) and ten calves (ages ranging from 6 to 7 months) shown to be seronegative by IFAT to *T. gondii* and *N. caninum* at the start of the experiment were used in this study.

Mature Swiss White mice of both sexes from *Toxoplasma* free colonies were used for inoculation of tissues obtained from orally infected sheep and cattle to determine the presence of viable *T. gondii* as described in Chapter 3 (3.8.2).

Cats of both sexes, under six months of age and seronegative for antibodies to *T. gondii*, obtained from a *Toxoplasma* free colony in the University of Newcastle (UK), were used to obtain oocysts (see Chapter 3).

5.2.2 Toxoplasma gondii

Toxoplasma gondii oocysts obtained from infection of seronegative cats (Chapter 3, 3.2.1) were used to dose the experimental animals.

Tachyzoites of the S48 strain prepared as described in Chapter 3 (3.2.2) were used as a source of *T. gondii* DNA for a positive control in the B1 PCR and tachyzoites of the RH strain were used as source of specific antigen for the serological analysis of infected mice in the ELISA (3.3.1).

5.2.3 Experimental design

The sheep and cattle were allocated into four groups as illustrated in Table 5. 1. Suspensions containing 10^3 and 10^5 sporulated *T. gondii* oocysts in PBS were administrated to the animals by gavage as described in Chapter 3 (3.2.1).

Table 5. 1 Experimental design

Group	Species	animal numbers	M3 oocyst dose
1	sheep	$S_1$1, $S_1$2, $S_1$3, $S_1$4	10^3
2	cattle	$C_2$1, $C_2$2, $C_2$3, $C_2$4	10^3
3	sheep	$S_3$1, $S_3$2, $S_3$3, $S_3$4, $S_3$5, $S_3$6	10^5
4	cattle	$C_4$1, $C_4$2, $C_4$3, $C_4$4, $C_4$5, $C_4$6	10^5

S_n**N**: sheep number **N** from group **n**, C_n**N**: cattle number **N** from group **n**

5.2.4 Parasite detection

To determine the presence of *T. gondii* within the tissues of the infected animals, half of the animals in each group were killed six weeks and the remainder six months after oral infection.

5.2.4.1 Examination of tissue sections by histopathology

Samples of brain, spinal cord, cardiac muscle, skeletal muscles (psoas, gracillis), liver, mesenteric lymph node, lung and kidney were collected. All the

samples were immediately fixed in 10% formal saline and processed as described in Chapter 3 (3.8).

5.2.4.2 Detection of T. gondii *from tissues by mouse inoculation*

Approximately 1 to 3g of infected tissue samples of brain, cardiac muscle and skeletal muscle (psoas, gracillis) were homogenized with trypsin in PBS, incubated at 37°C for 60 minutes, large particulate matter removed and then intraperitoneally inoculated into mice (Chapter 3, 3.8.3). Three mice were inoculated per tissue sample. Mice were inspected daily for *T. gondii* infection and those which appeared ill (symptoms described earlier in Chapter 3, 3.8.3) were killed and a sample of peritoneal exudate removed to examine for the presence of tachyzoites. Serum was collected from surviving mice 8 weeks after inoculation and tested for the presence of *Toxoplasma* antibodies by IgG ELISA as described in Chapter 3.

5.2.4.3 Detection of T. gondii *in infected tissues by polymerase chain reaction*

Approximately 5g (from animals given 10^3 oocysts) and 100g (from animals given 10^5 oocysts) of tissue samples of brain, cardiac muscle and skeletal muscle (psoas and gracillis) were stored at -20°C prior to extraction of DNA for PCR analysis. Approximately 2g of each sample were analysed and when a positive result was obtained the remainder of the sample was not tested. The PCR product was confirmed to be *T. gondii* DNA by Southern blotting and immunological detection of bound DIG labelled probes to *T. gondii* DNA described in Chapter 3 (3.5.2).

5.3 Results

5.3.1 Clinical response

Confirmation of infection following administration of oocysts was defined by a febrile response and specific seroconversion. Details of the results are described in Chapter 6: Clinical and humoral immune responses of sheep and cattle to *Toxoplasma gondii* infection.

5.3.2 Examination of tissues at post-mortem

Post-mortem examination did not reveal macroscopic lesions in any of the animals killed at either six weeks or six months following infection with either 10^3 or 10^5 *T. gondii* oocysts.

5.3.3 Examination of tissues by histopathology

5.3.3.1 Oocyst dose: 10^3

5.3.3.1.1 Examination of tissue sections at 6 weeks after infection by histopathology

Six weeks following infection, no obvious histopathological changes were detected in samples of brain, heart and skeletal muscle taken from the cattle orally infected with 10^3 *T. gondii* oocysts of the M3 isolate. However, cyst-like structures were detected in heart samples from sheep $S_1$4 and in skeletal muscle in sheep $S_1$3. There was no evidence of any inflammatory reaction around the cysts. Marked cuffing of many blood vessels was seen in the brain (mesencephalo) of sheep $S_1$3.

As there was little evidence of infection in the tissues of either sheep or cattle six weeks after infection, the tissues from the other animals were not examined after six months.

5.3.3.2 Oocyst dose: 10^5

5.3.3.2.1 Examination of tissue sections at 6 weeks after infection by histopathology

T. gondii-like cysts were seen in heart samples of two of the sheep infected with 10^5 oocysts of the M3 isolate ($S_3$1 and $S_3$2) and in gracillis muscle in sheep $S_3$1 and $S_3$3. There was no evidence of any inflammatory reaction around the cysts in these samples. Disseminated nodules with no necrotic center were seen in the brain in the sheep $S_3$1, and an accumulation of cells was located around blood vessels. Necrotic areas with glial cells were described in the brain of one of the sheep ($S_3$3).

No histopathological changes were seen in cattle given 10^5 oocysts.

5.3.3.2.2 Examination of tissue sections at 6 months after infection by histopathology

Cyst-like structures were seen in sections of skeletal muscles as well as in heart samples from the three sheep ($S_3$4, $S_3$5 and $S_3$6). Again there was no evidence of any inflammatory reaction around the cysts present in these samples.

No histopathological changes were seen in sections of tissues from the three cattle.

5.3.4 Detection of *T. gondii* from tissues by mouse inoculation

5.3.4.1 Oocyst dose: 10^3

5.3.4.1.1 Examination of tissues at 6 weeks after infection

Table 5. 2 shows the results obtained from the bioassays in mice carried out after i.p. inoculation of Swiss White mice with tissue samples from two sheep ($S_1$3 and $S_1$4) and two cattle ($C_2$1 and $C_2$3) killed 6 weeks after infection with *T. gondii* oocysts.

Viable *T. gondii* was detected in the mice inoculated with heart sample from one of the sheep ($S_1$4). In this case the three mice inoculated with the sample of heart tissue showed symptoms of acute infection but they recovered and developed a high titre of specific IgG against *T. gondii*.

Acute infection was not detected in any of the mice inoculated with cattle tissue samples.

Table 5. 2 Viable *T. gondii* detected in tissue samples from sheep and cattle orally infected with 10^3 *T. gondii* oocysts and killed 6 weeks after infection

Animal	Detection of infection	Tissue samples			
		brain	heart	psoas	gracillis
$S_1$3	acute	0/3	0/3	0/3	0/3
	ELISA	0/3	0/3	0/3	0/3
$S_1$4	acute	0/3	0/3	0/3	0/3
	ELISA	0/3	101%, 79%, 71%	0/3	0/3
$C_2$1	acute	0/3	0/3	0/3	0/3
	ELISA	0/3	0/3	0/3	0/3
$C_2$3	acute	0/3	0/3	0/3	0/3
	ELISA	0/3	0/3	0/3	0/3

Three mice were inoculated with each tissue sample. The term **acute** refers to the mice culled due to acute toxoplasmosis defined by signs of febrile response and presence of tachyzoites within peritoneal exudate. **ELISA** refers to the mice which survived and were bled 8 weeks after inoculation. The presence of specific *T. gondii* antibodies was determined by an ELISA detecting specific IgG responses, and the data were expressed as a percentage of a standard high titre positive serum. A positive result was taken to be any reading above 10% OD (Chapter 3, 3.6.2). 0/3 means no reaction was detected from any of the three mice tested.

5.3.4.2 Oocyst dose: 10^5

5.3.4.2.1 Examination of tissues at 6 weeks after infection

Table 5. 3 shows the results obtained after i.p. inoculation of Swiss White mice with tissue samples from 3 sheep ($S_3$1, $S_3$2, $S_3$3) and 3 cattle ($C_4$1, $C_4$2, $C_4$3) killed six weeks following oral infection with 10^5 *T. gondii* oocysts of the M3 isolate.

Viable parasites were frequently detected from tissue samples of the sheep. The four different tissue samples taken from one of the sheep ($S_3$2) resulted in an acute infection in mice and tachyzoites were seen in the peritoneal fluid. Viable parasites were detected from the other two sheep ($S_3$1 and $S_3$3), in three different tissue samples taken (brain, heart and psoas). Despite evidence that the mice were clearly infected with *T. gondii,* specific IgG was only detected in two of the mice inoculated with tissue samples (brain and psoas) from one of the sheep ($S_3$2).

It can be seen from Table 5. 3 that acute infection was not detected in any of the mice i.p. inoculated with cattle tissues, but specific IgG against *T. gondii* was detected using ELISA in one of the mice infected with brain tissue of one of the cattle ($C_4$2).

Table 5. 3 Viable *T. gondii* detected in tissue samples from sheep and cattle orally infected with 10^5 *T. gondii* oocysts and killed 6 weeks after infection

Animal	Detection of infection	Tissues sample			
		Brain	Heart	Psoas	Gracillis
$S_3$1	acute	2/3	3/3	2/3	0/3
	ELISA	0/1	-	0/1	0/3
$S_3$2	acute	1/3	3/3	1/3	2/3
	ELISA	38%/2	-	103%/2	0/1
$S_3$3	acute	1/3	3/3	1/3	0/3
	ELISA	0/2	-	0/2	0/3
$C_4$1	acute	0/3	0/3	0/3	0/3
	ELISA	0/3	0/3	0/3	0/3
$C_4$2	acute	0/3	0/3	0/3	0/3
	ELISA	45%/3	0/3	0/3	0/3
$C_4$3	acute	0/3	0/3	0/3	0/3
	ELISA	0/3	0/3	0/3	0/3

Three mice were inoculated with each tissue sample. The term **acute** refers to the mice culled due to acute toxoplasmosis defined by signs of febrile response and presence of tachyzoites within peritoneal exudate. **ELISA** refers to the mice which survived and were bled 8 weeks after inoculation. The presence of specific *T. gondii* antibodies was determined by an ELISA detecting specific IgG responses, and the data were expressed as a percentage of a standard high titre positive serum. A positive result was taken to be any reading above 10% OD (Chapter 3, 3.6.2). 0/3 means no reaction was detected from any of the three mice tested.

5.3.4.2.2 Examination of tissues at 6 months after infection

Table 5. 4 illustrates the results obtained after i.p. inoculation of Swiss White mice with tissue samples from sheep ($S_3$4, $S_3$5, $S_3$6) and cattle (C_4 4, $C_4$5, $C_4$6) killed six months following oral infection with 10^5 *T. gondii* oocysts of the M3 isolate.

Viable parasites were detected from sheep samples of heart (3/3), brain tissue ($S_3$4 and $S_3$5), and skeletal muscle ($S_3$4, $S_3$5). Specific IgG antibodies were not detected in any of the mice inoculated i.p. with tissue samples.

There was no evidence of *T. gondii* in any of the tissues collected 6 months after infection in the group of cattle.

Table 5. 4 Viable *T. gondii* detected in tissue samples from sheep and cattle orally infected with 10^5 *T. gondii* oocysts and killed 6 months after infection

Animal	Detection of infection	Tissue samples			
		Brain	Heart	Psoas	Gracillis
$S_3$4	acute	3/3	3/3	1/3	0/3
	ELISA	0/3	0/3	0/2	0/3
$S_3$5	acute	2/3	3/3	0/3	1/3
	ELISA	0/1	-	0/3	0/2
$S_3$6	acute	0/3	2/3	0/3	0/3
	ELISA	0/3	0/1	0/3	0/3
$C_4$4	acute	0/3	0/3	0/3	0/3
	ELISA	0/3	0/3	0/3	0/3
$C_4$5	acute	0/3	0/3	0/3	0/3
	ELISA	0/3	0/3	0/3	0/3
$C_4$6	acute	0/3	0/3	0/3	0/3
	ELISA	0/3	0/3	0/3	0/3

Three mice were inoculated with each tissue sample. The term **acute** refers to the mice culled due to acute toxoplasmosis defined by signs of febrile response and presence of tachyzoites within peritoneal exudate. **ELISA** refers to the mice which survived and were bled 8 weeks after inoculation. The presence of specific *T. gondii* antibodies was determined by an ELISA detecting specific IgG responses, and the data were expressed as a percentage of a standard high titre positive serum. A positive result was taken to be any reading above 10% OD (Chapter 3, 3.6.2). 0/3 means no reaction was detected from any of the three mice tested.

5.3.5 Detection of *T. gondii* in infected tissues by PCR

Table 5. 5 summarizes results obtained from PCR amplification of the B1 gene of *T. gondii* from tissue samples taken from sheep and cattle, which were infected either with 10^3 or 10^5 sporulated *T. gondii* oocysts. The samples were taken at post-mortem examination either six weeks or six months after the animals had been infected. Figure 5. 1 shows that the B1 gene of *T. gondii* was amplified from tissue samples of sheep orally infected with *T. gondii* oocysts. Figure 5. 2 shows the nitrocellulose membrane revealing the immunological detection of bound DIG labelled probes to *T. gondii* DNA as specific confirmation of the product detected.

Specific DNA was detected in tissue samples from sheep. *Toxoplasma gondii* DNA was more frequently detected in tissues from sheep given the higher infective dose of oocysts (10^5) compared to the 10^3 dose. Only the sample of heart tissue from one of the sheep (S_14) infected with 10^3 oocyst gave a positive result with the B1 PCR.

Specific *T. gondii* DNA was not detected on any occasion from the samples taken from cattle.

There was not much difference in the frequency of detection or tissue distribution of *T. gondii* in sheep at six weeks compared to six months following infection with 10^5 oocysts. Specific DNA was detected and amplified from brain samples of all the sheep at six weeks and six months following infection with 10^5 oocysts, from heart tissue sample of two sheep (S_31 and S_32) killed six weeks after infection and from the heart sample of one sheep (S_34) killed six months after infection with 10^5 oocysts.

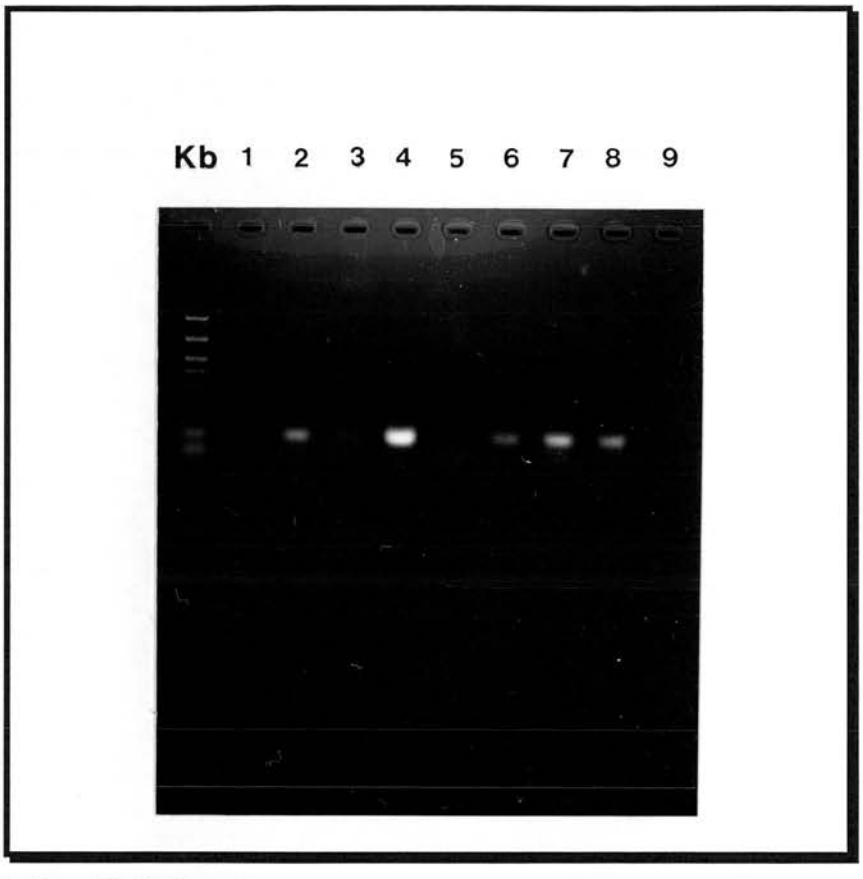
Table 5. 5 *T. gondii* DNA detection and amplification of the B1 gene of *T. gondii* in tissue samples by the B1 PCR from sheep and cattle undergoing primary oral infection with either 10^3 or 10^5 M3 *T. gondii* oocysts and killed either 6 weeks or 6 months after infection.

Animal	M3 oocyst dose	time after infection	Tissue samples			
			brain	heart	psoas	gracillis
$S_1$3	10^3	6 weeks	-	-	-	-
$S_1$4	10^3	6 weeks	-	❖	-	-
$C_2$1	10^3	6 weeks	-	-	-	-
$C_2$4	10^3	6 weeks	-	-	-	-
$S_3$1	10^5	6 weeks	❖	❖	-	-
$S_3$2	10^5	6 weeks	❖	❖	-	-
$S_3$3	10^5	6 weeks	❖	-	-	-
$C_4$1	10^5	6 weeks	-	-	-	-
$C_4$2	10^5	6 weeks	-	-	-	-
$C_4$3	10^5	6 weeks	-	-	-	-
$S_3$4	10^5	6 months	❖	❖	-	-
$S_3$5	10^5	6 months	❖	-	-	-
$S_3$6	10^5	6 months	❖	-	-	-
$C_4$4	10^5	6 months	-	-	-	-
$C_4$5	10^5	6 months	-	-	-	-
$C_4$6	10^5	6 months	-	-	-	-

❖: detection and amplification of *T. gondii* DNA in PBM cells by the B1 PCR

-: no detection of *T. gondii* DNA in PBM cells by the B1 PCR

Figure 5. 1 *T. gondii* B1 gene detected and amplified by PCR from tissue samples of sheep undergoing primary oral infection with *T. gondii* oocysts



kb: DNA ladder of 50-1000 bp with a concentration in each band of approximately 50 ng/ml

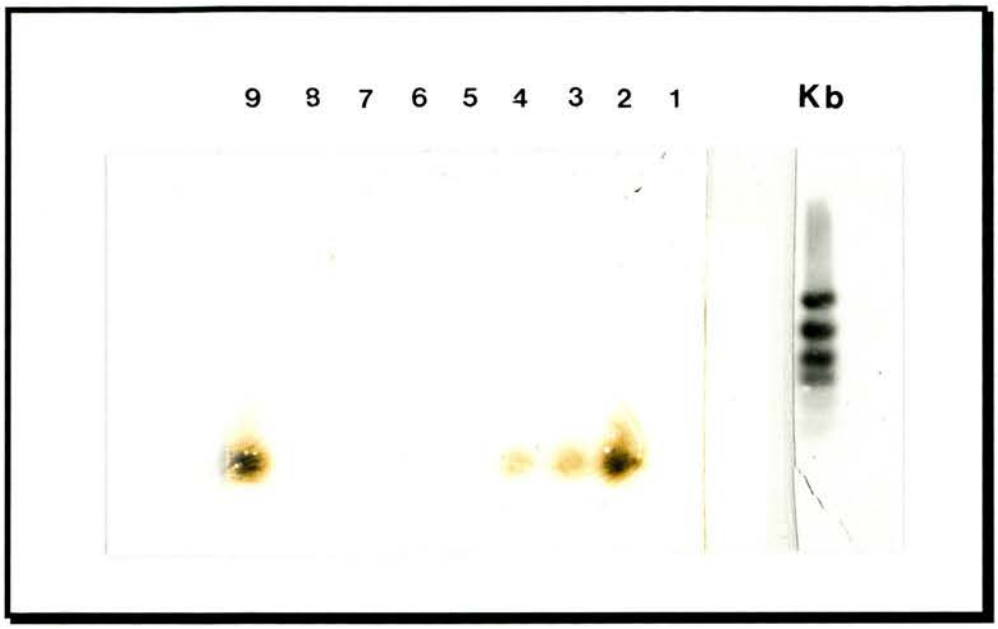
Lane 1: negative control consisting of UV sterilised distilled water

Lane 2: positive control consisting of 96 bp product of the B1 gene of *T. gondii*

Lane 3 to lane 9: B1 gene of *T. gondii* amplified from tissue samples of infected sheep using P_2 and P_3 primers

Lane 3: $S_3$1-brain, Lane 4: $S_3$2-heart, Lane 5: $S_1$4-psoas, Lane 6: $S_3$3-psoas, Lane 7: $S_3$5-gracillis, Lane 8: $S_3$6-gracillis and Lane 9: $S_3$4- heart.

Figure 5. 2 *T. gondii* B1 gene by Southern blot from agarose gel



Lane 1: negative control consisting of UV sterilised distilled water

Lane 2: positive control consisting of 96 bp product of the B1 gene of *T. gondii*

Lane 3 to lane 9: B1 gene of *T. gondii* amplified from tissue samples of infected sheep using P_2 and P_3 primers

Lane 3: $S_3$1-brain, Lane 4: $S_3$2-heart, Lane 5: $S_1$4-psoas, Lane 6: $S_3$3-psoas, Lane 7: $S_3$5-gracillis, Lane 8: $S_3$6-gracillis and Lane 9: $S_3$4- heart.

kb ladder: DNA ladder of 50-1000 bp with a concentration in each band of approximately 50 ng/ml

5.3.6 Comparison of bioassay in mice with B1 PCR

Table 5. 6 illustrates the results from the bioassay in mice compared with the B1 PCR on samples taken from the infected animals at either six weeks or six months after infection.

Using the mouse inoculation technique viable *T. gondii* parasites were detected in 9 samples which were also positive by the B1 PCR technique. 43 samples analysed were negative by both techniques. *T. gondii* was detected by mouse inoculation in 11 tissue samples but *T. gondii* DNA was not detected from these samples when analysed by the B1 PCR. Only in one sample specific *Toxoplasma* DNA was detected by PCR but *T. gondii* was not detected by mouse inoculation.

Table 5. 6 Comparison of bioassay in mice and the B1 PCR for detection of *T. gondii* in tissue samples from sheep and cattle infected with *T. gondii* oocysts.[1]

Animal	M3 oocyst dose	Time after infection	Tissue samples							
			brain		heart		psoas		gracillis	
			MI	PCR	MI	PCR	MI	PCR	MI	PCR
$S_1$3	10^3	6 weeks	-	-	-	-	-	-	-	-
$S_1$4	10^3	6 weeks	-	-	❖	❖	-	-	-	-
$C_2$1	10^3	6 weeks	-	-	-	-	-	-	-	-
$C_2$4	10^3	6 weeks	-	-	-	-	-	-	-	-
$S_3$1	10^5	6 weeks	❖	❖	❖	❖	❖	-	❖	-
$S_3$2	10^5	6 weeks	❖	❖	❖	❖	❖	-	❖	-
$S_3$3	10^5	6 weeks	❖	❖	❖	-	❖	-	-	-
$C_4$1	10^5	6 weeks	-	-	-	-	-	-	-	-
$C_4$2	10^5	6 weeks	❖	-	-	-	-	-	-	-
$C_4$3	10^5	6 weeks	-	-	-	-	-	-	-	-
$S_3$4	10^5	6 months	❖	❖	❖	❖	❖	-	-	-
$S_3$5	10^5	6 months	❖	❖	❖	-	-	-	❖	-
$S_3$6	10^5	6 months	-	❖	❖	-	-	-	-	-
$C_4$4	10^5	6 months	-	-	-	-	-	-	-	-
$C_4$5	10^5	6 months	-	-	-	-	-	-	-	-
$C_4$6	10^5	6 months	-	-	-	-	-	-	-	-

[1] MI: bioassay in mice (i.p. mouse inoculation)

5.4 Discussion

The main finding from this study was that *T. gondii* could be detected in the tissues of sheep whereas it was not possible, under the conditions of this experiment, to detect *T. gondii* in the tissues of cattle.

Infection rates in food animals vary in many parts of the world as indicated by serological surveys (Fayer, 1981; Dubey, 1986a, b; Dubey and Towle, 1986). There are reports in the literature supporting the hypothesis that cattle are not a favoured host for *T. gondii* and are able to reduce the number or even to get rid of *Toxoplasma* cysts in their tissues (Sanger *et al.*, 1953; Rawal, 1959; Jacobs *et al.*, 1960b, Meyer, 1963; Zardi *et al.*, 1964; Work, 1967; Catár *et al.*, 1969; Dubey and Streitel, 1976). The presence of *T. gondii* cysts in the tissues of naturally and experimentally infected sheep have been reported (Jacobs *et al.*, 1960a, b; Jacobs *et al.*, 1963; Jacobs and Hartley, 1964; Dubey and Sharma, 1980). *Toxoplasma gondii* has been detected from soft tissues such as brain, cardiac muscle, skeletal muscle, small intestine, liver and diaphragm. The brain is the most frequently infected tissue in either aborted foetuses or live animals born to infected ewes and has been suggested as the organ of choice for isolating *T. gondii* for diagnostic purposes (Uggla *et al.*, 1987).

Confirming the findings of the experiment described Chapter 4 of this thesis, *T. gondii* was more frequently detected in sheep given 10^5 compared to 10^3 oocysts. In sheep given 10^5, *T. gondii* was more frequently detected in brain and heart tissues and was not demonstrated to be present in skeletal muscle. Examination of tissues at either 6 weeks or 6 months after infection did not appear to affect the distribution of *T. gondii* within the tissue sampled.

As discussed in Chapter 4 the oocyst dose does affect the frequency of *T. gondii* detection within ovine tissues (Dubey and Sharma, 1980). The results

from the experiments described in Chapter 4 and 5 of this thesis suggest that the brain and heart are the favoured site for detection of *T. gondii* in experimentally infected adult sheep. It has been demonstrated that although tissue cysts appear to have predilection for the CNS, they may also be found in skeletal and cardiac muscle (Jacobs *et al.*, 1960a) and that intact cysts may persist for the life time of the host (Dubey, 1977). However, Dubey and Sharma (1980) suggested that because in their experiment the parasite was isolated consistently from skeletal muscle of sheep orally infected with *Toxoplasma* oocysts, this may be a favoured site for *T. gondii*. It has been reported that brain from congenitally infected sheep is the most heavily infected tissue (Watson and Beverley, 1971; Dubey, 1984; Uggla *et al.*, 1987).

Mature cysts will develop in chronically infected mice at 6-8 weeks after infection (McHugh *et al.*, 1994). During a chronic *Toxoplasma* infection, similar to the present study, the parasite is not only in the form of encysted bradyzoite but also individual bradyzoites which can escape from cysts without complete cyst wall disruption (Santoro *et al.*, 1985; Wong and Remington, 1993). Therefore it is not possible using the detection methods described in this study to state the parasite stage detected. However, since the postmortem examinations took place either at 6 weeks or 6 months after infection, it is likely that the bradyzoite stage was detected.

Examination of animal tissues for the detection of *T. gondii* can be done using various techniques which differ in their sensitivity and specificity.

There are few reports in the literature concerning pathological changes following a primary infection of adult immunocompetent animals. The majority of reports describe pathological changes following infection of the immunologically immature foetus (Hartley and Kater, 1963; Beverley *et al.*, 1971; Buxton *et al.*, 1982).

The lesions following *Toxoplasma* infection in adult animals occur most commonly in the nervous system, and lymph nodes. In 1961 Koestner and

Cole compared the central nervous system lesions from cases of both natural and experimentally infected sheep and cattle. They reported that common feature of *Toxoplasma* infection of the CNS in these species is characterized by focal necrosis and vascular damage in animals with acute infection and by glial nodules, repair and vascular mineralization in animals with chronic infection. Buxton and colleagues (1981) outlined the pathological changes in ovine popliteal lymph nodes after subcutaneous inoculation of sheep with tissue cysts of *T. gondii.* These included gross enlargement, loss of architecture, haemorrhaging, some necrosis and sinuses full of plasma cells and numerous blast cells. The changes in lymph nodes from sheep immune to *T. gondii* were similar but less striking.

Macroscopic lesions are difficult to find in immunocompetent adult sheep (Garnham and Lainson, 1960; McErlan, 1974; Sharma and Dubey, 1981; Dubey, 1984) or cattle (Sanger *et al.*, 1953). Reports of cattle experimentally infected with *T. gondii* (similar to the study discussed in this chapter) revealed no macroscopic or histopathological changes at post mortem examination (Costa *et al.*, 1977; Beverley *et al.*, 1977).

Tachyzoites begin to disappear from visceral tissues in the third week following infection and may be localised as bradyzoites within cysts in neural and muscular tissues (Dubey and Frenkel, 1974). Some residual cerebral lesions have been described in brains of hosts which survived acute *Toxoplasma* infection, consisting of microglial nodules alongside more extensive hyperplasia of perithelial cells and perivascular fibrosis. Inflammation is not usually associated with cysts and only in cases where cysts have ruptured releasing parasites does, a severe inflammation occurs along with local necrosis (Dubey, 1977). At this phase of *Toxoplasma* infection, tachyzoites are rarely seen, and cysts 30µm diameter with a wall of acidophilic material approximately 0.5µm thick, located in areas away from lesions, may be the only form seen (Jubb *et al.*, 1993).

It may be difficult to find *T. gondii* in sections of animal tissues because of the low number of organisms present specially when looking at tissues from large animal species. Moreover, because tissue cyst-like structures from *Sarcocystis* spp., *N. caninum* and *T. gondii* can appear morphologically similar in sections stained with H&E, an immunohistochemistry technique may help to specifically stain parasites in tissue section from infected animals (Uggla *et al.*, 1987; Lindsay and Dubey, 1989; Cole *et al.*, 1993). In summary examination of tissue sections by H&E is neither specific or sensitive enough as a technique to examine for the presence of *T. gondii* which may be sparsely distributed within tissues of large animal species (Dubey and Streitel, 1976; Beverley *et al.*, 1977; Sharma and Dubey, 1981; Dubey and Thulliez, 1993).

A more sensitive and specific technique involves the inoculation of tissue samples into mice which are extremely susceptible to infection with *T. gondii*, whereas *Sarcocystis* spp. is not infective and *N. caninum* is rarely infective to immunocompetent mice (Dubey *et al.*, 1988b; 1989). Larger volumes of tissues (500g or more) can be fed to naive cats than can be used to infect mice (Dubey and Thulliez, 1993). Therefore the former would be the ideal animal for bioassay detection but this is clearly impractical as it is extremely difficult and expensive to obtain *T. gondii* naive cats.

Inoculation of tissues into mice is a widely used method of bioassay due to the sensitivity of mice to *T. gondii* infection. However, isolation of the parasite by bioassay in mice is time consuming and potentially hazardous for the operator. An increasingly popular detection method involving amplification of the *T. gondii* B1 gene by PCR which is able to detect small amounts of DNA (0.05 pg). This technique is known to be a sensitive method for the diagnosis of *Toxoplasma* infection, and the results agree closely with those from mouse inoculation (Wastling *et al.*, 1993; Greig *et al*, 1993; Steuber *et al.*, 1995). The results from this study generally show good agreement between the two assay systems, although the mouse inoculation assay detects live parasites only,

whereas the PCR assay can detect specific DNA which may arise from either live or dead parasite material.

One of the major difficulties in detection of *T. gondii* in tissue from large animal species, such as sheep and cattle used in this study, is the limitation of the size of sample able to be examined. While it was not possible to detect *T. gondii* in cattle using the samples and detection methods described in this study, it is possible that the parasite may be present in unexamined tissues. The only way to demonstrate that *T. gondii* did not persist in cattle tissues would be to examine the whole animal which would be clearly impractical.

5.5 Conclusions

The results from this study show that *T. gondii* is more frequently detected in the tissues of infected sheep compared to cattle.

It may also be inferred that an increase in the infective oocyst dose augments the distribution of *T. gondii* within ovine tissues.

Finally, sample error is an important element to take into consideration when attempting to detect *T. gondii* within tissues from large animal species. Therefore the absence of macroscopic or histopathological alterations of organs, no tissue cysts seen on histopathological sections and no parasite detection by either bioassay in susceptible laboratory animals or PCR do not exclude the possibility of infection in large animals such as sheep and cattle.

The reasons why cattle may be better able to control *T. gondii* infection compared to sheep are unknown. A key factor could be the relative efficiency of the host immune response provoked by *T. gondii* within sheep and cattle.

The following two chapters describe studies on the host immune response in sheep and cattle following oral infection with *T. gondii* oocysts.

Chapter 6

Comparison of clinical and humoral immune responses of sheep and cattle to a primary *Toxoplasma gondii* infection

Aims

To compare the clinical response, parasitaemia and humoral immune response to a primary *T. gondii* infection in sheep and cattle

6. Comparison of clinical and humoral immune responses of sheep and cattle to a primary *Toxoplasma gondii* infection

6.1 Introduction

Toxoplasma gondii infection in normal immunocompetent hosts is generally asymptomatic (Dubey and Beattie, 1988).

However, it is known that *Toxoplasma* infection in non-pregnant immunocompetent sheep (Dubey and Towle, 1986) and cattle can provoke a febrile response, dyspnoea and anorexia within the first two weeks of infection before returning to normal (Dubey, 1986b).

In sheep, following experimental infection with *T. gondii*, specific antibody titres rise 2-3 weeks after infection, remaining elevated for several years (Blewett *et al.*, 1983). Under field conditions this antibody response is often used as an aid to diagnosis (Munday and Corbould, 1971; Blewett *et al.*, 1983; Trees *et al.*, 1989). It is interesting to note that reinfection of sheep with either *T. gondii* tissue cysts (Blewett *et al.*, 1983) or oocysts (McColgan *et al.*, 1988) does not elicit an increase in antibody titre.

There are few reports in the literature concerning detection of *T. gondii* specific antibodies in cattle. This may be because *T. gondii* is not thought to be a major cause of disease in cattle (Dubey, 1986b) but also there have been reports that it is difficult to accurately determine specific antibody titres in cattle due to a problem with non-specific activity in sera (Dubey and Streitel, 1976; Dubey, 1985; Dubey *et al.*, 1985). Even so specific antibody responses have been described in cattle experimentally infected with *T. gondii* (Costa *et*

al., 1977; Beverley *et al.*, 1977; Munday, 1978; Dubey, 1986b). However, is seems likely that sheep are more susceptible to *T. gondii* infection than cattle as research has shown that sheep grazing on a pasture alongside cattle developed significant antibody titres to *T. gondii* as measured by IFAT while none of the calves became sero-positive (Munday and Corbould, 1971).

Although a humoral immune response is clearly induced as a result of *T. gondii* infection, its importance in protective immunity is unclear. Passive transfer of immune serum (Krahenbuhl *et al.*, 1972; Pavia, 1986) or monoclonal antibodies (Johnson *et al.*, 1983) only confer partial protection against infection in mice. It is known that specific antibodies, in the presence of complement, will lyse extracellular tachyzoites (Schreiber and Feldman, 1980) and that they may also play a role in preventing invasion of host cells in two ways. Antibody may block the activity of secretory-excretory substances which are thought to aid parasite entry into host cells (Schwartzman, 1986) and tachyzoites which are opsonized by antibody or complement are unable to prevent fusion of their parasitophorous vacuoles with host cell lysosomes and are therefore killed by normal macrophage destruction mechanisms (Joiner *et al.*, 1990).

The aims of the study described in this chapter were to compare the temperature responses and specific antibody titres in sheep and cattle following a primary oral infection with *T. gondii* oocysts.

6.2 Materials and methods

6.2.1 Animals

Ten adult sheep (ages ranging from 2 to 5 years) and ten calves (ages ranging from 6 to 7 months) shown to be seronegative by IFAT to *T. gondii* and *N. caninum* at the start of the experiment were used in this study.

Mature Swiss White mice of both sexes from *Toxoplasma* free colonies were used to passage and maintain the S48 strain of *T. gondii* tachyzoites as described in Chapter 3 (3.1.3, 3.2).

Cats of both sexes, under six months of age and seronegative by IFAT for antibodies to *T. gondii*, obtained from a *Toxoplasma* free colony in the University of Newcastle (UK), were used in this study.

6.2.2 *Toxoplasma gondii*

Oocysts of the M3 isolate were prepared from the faeces of seronegative cats which had been fed *T. gondii* tissue cysts as described in Chapter 3 (3.2.1.3).

Tachyzoites of the S48 strain prepared as described in Chapter 3 were used as antigen in the serological analyses of infected animals and to extract *T. gondii* DNA as a positive control for the B1 PCR.

6.2.3 Experimental design

Sheep and cattle were allocated into four groups as described in Table 6. 1. Doses of either 10^3 or 10^5 *T. gondii* oocysts were administrated by gavage. These are the same experimental groups used in Chapter 5 of this thesis.

Table 6. 1 Experimental design

Group	Species	Animal numbers	M3 oocysts
1	sheep	$S_1$1, $S_1$2, $S_1$3, $S_1$4	10^3
2	cattle	$C_2$1, $C_2$2, $C_2$3, $C_2$4	10^3
3	sheep	$S_3$1, $S_3$2, $S_3$3, $S_3$4, $S_3$5, $S_3$6	10^5
4	cattle	$C_4$1, $C_4$2, $C_4$3, $C_4$4, $C_4$5, $C_4$6	10^5

S_n**N**: sheep number **N** from group **n**, C_n**N**: cattle number **N** from group **n**

6.2.4 Clinical response

6.2.4.1 Rectal temperatures

Rectal temperatures of sheep and cattle were recorded daily for the 3 days before and 14 days after oral dosing with a clinical electronic thermometer.

6.2.4.2 Parasitaemia

Blood samples were taken from the jugular vein of sheep and cattle daily from 3 days prior to infection until 14 days after infection. Peripheral blood mononuclear cells extracted as described in Chapter 3: Materials and Methods. These samples were analysed by the B1 PCR to detect and amplify *Toxoplasma* specific DNA following the procedure described in Chapter 3: (3.5.2.).

6.2.5 Humoral immune response

Blood samples were collected from the jugular vein of sheep and cattle into preservative-free evacuated blood collection tubes, and allowed to clot. Following retraction of the clot and centrifugation at 500*g* for 15 minute serum was removed, aliquoted and stored at -20° C until required. Serum samples were collected from both sheep and cattle for 4 days prior to infection, and then every two days for 3 weeks (animals given 10^3 oocysts) or 2 weeks (animals given 10^5 oocysts). Thereafter samples were collected weekly and then monthly for 1 year (animals given 10^3 oocysts) and 6 months (animals given 10^5 oocysts), (See Appendix Chapter 6 for further details).

6.2.5.1 Indirect Fluorescent Antibody Test

Serum samples from experimental sheep and cattle were monitored for the presence of *T. gondii* specific IgG antibodies using the IFAT (Chapter 3, 3.6.1).

In brief, a suspension of formalinised tachyzoites was layered onto slides, air dried and fixed in methanol. After washing with PBS, diluted test sera were added to the slides, incubated for 30 minutes and then washed with PBS. The slides were incubated for further 30 minutes with a second antiserum conjugated with FITC, washed and viewed with UV light under an Olympus BX50 microscope using a U-MNB filter cube with a ×40 objective.

6.3 Results

6.3.1 Clinical response

6.3.1.1 Rectal temperatures

The first clinical signs in the sheep and cattle occurred during the first week after infection when all the animals showed an increase in respiratory rate, mild anorexia and a small febrile response when compared with pre-infection observations. All the data corresponding to the rectal temperatures were analysed taking the mean of the temperature values for each group recorded before infection on day 0 as a base line.

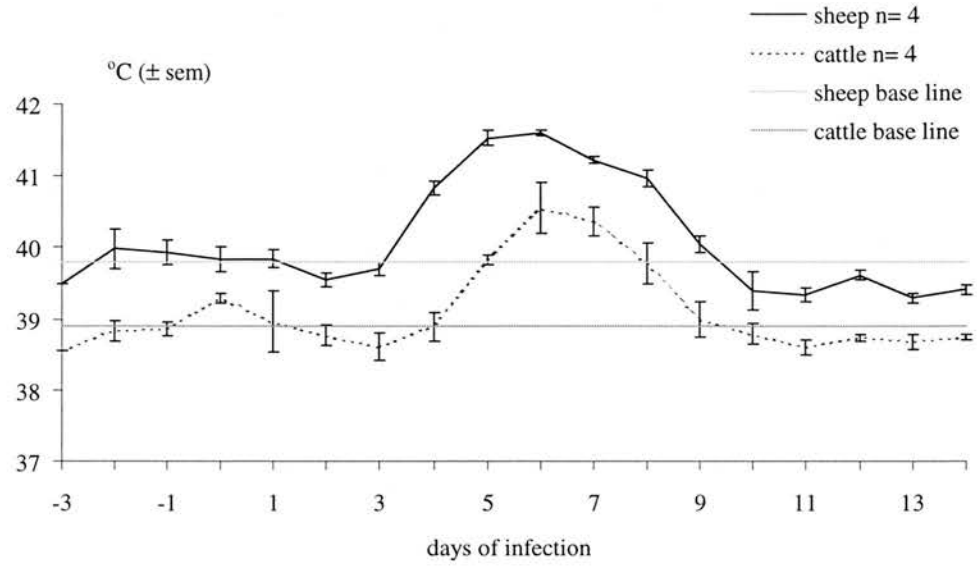
Figure 6. 1 shows the mean rectal temperatures recorded from the groups of sheep and cattle for a period of 14 days after oral infection. The sheep infected with 10^3 oocysts showed a significant febrile response ($P< 0.01$ Student's t-test) compared to base line values on four, five, six and seven days after infection. A maximum mean temperature was reached on day six and values returned to normal by day eight. Cattle showed a rise in temperature on day five, significantly later ($P<0.01$ Student's t-test) than the sheep group. The mean temperature in the cattle reached a peak on day 6 and returned to normal values on day 9 (Figure 6.1.a).

When the infective dose was increased to 10^5 oocysts, the sheep developed a significant pyrexia ($P< 0.01$ Student's t-test) compared to the base line values on day three, four, five, six, seven and eight after infection. They reached a peak on day four before returning to normal values by day eight. The cattle also showed a febrile response on day three ($P< 0.01$ Student's t-test), reaching a peak on day five post infection, and returning to base line values on day 8 (Figure 6.1.b).

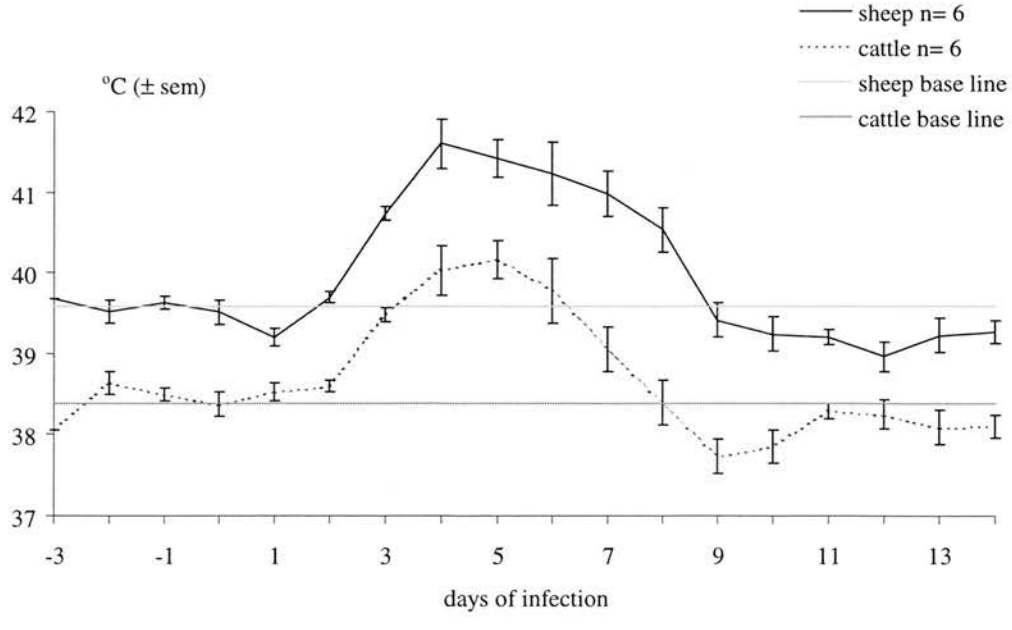
In summary, although both 10^3 and 10^5 *T. gondii* oocysts induced pyrexia in all the animals, the febrile response started earlier in the sheep and lasted longer when compared to the cattle.

Figure 6. 1 Mean rectal temperature response of sheep and cattle following primary oral infection with sporulated *T. gondii* oocysts.

a) dose: 10^3 oocysts, base line represented with a continuous line (sheep: 39.81°C, cattle: 38.88°C)



b) dose: 10^5 oocysts, base line represented with a continuous line (sheep: 39.58°C, cattle: 38.39°C)



6.3.1.2 Parasitaemia

Specific *T. gondii* DNA was amplified by the B1 PCR from PBM cells taken from sheep and cattle, both before and after infection. The results obtained are summarised on Table 6. 2 and Table 6. 3. *T. gondii* DNA was not amplified from any of the samples taken before infection (the samples on day 0 were taken before the infection was given).

Table 6. 2 illustrates the results from the sheep and cattle infected with 10^3 oocysts. *T. gondii* DNA was only detected in PBM cells from 2 of the sheep infected with 10^3 oocysts on days 7 and 8 post infection. Positive results were not recorded in any of the bovine samples.

Table 6. 2 Detection of *T. gondii* DNA by B1 PCR in the PBM cells of sheep and cattle infected with 10^3 *T. gondii* oocysts

Animal	days of infection														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
$S_1$1	-	-	-	-	-	-	-	✤	✤	-	-	-	-	-	-
$S_1$2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_1$3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_1$4	-	-	-	-	-	-	-	✤	✤	-	-	-	-	-	-
$C_2$1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_2$2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_2$3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_2$4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

✤: detection and amplification of *T. gondii* DNA in PBM cells by the B1 PCR

-: no detection of *T. gondii* DNA in PBM cells by the B1 PCR

Table 6. 3 shows the results from the sheep and cattle infected with 10^5 oocysts. Specific DNA was detected and amplified in 4 of the 6 sheep and in three sheep on day 7 after infection. The earliest detection of *T. gondii* was on day 2 and the latest time of parasite detection was on day 14. In the group of cattle *Toxoplasma* DNA was detected earlier with 5 of the 6 cattle being positive on day 2 after infection and 3 positive on day 5 and one on day 13.

Table 6. 3. Detection of *T. gondii* DNA by B1 PCR in the PBM cells of sheep and cattle infected with 10^5 *T. gondii* oocysts

Animal	days of infection														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
$S_3$1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_3$2	-	-	-	-	-	-	❖	❖	-	-	-	-	-	-	-
$S_3$3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_3$4	-	-	-	-	-	-	-	❖	-	-	-	-	-	-	-
$S_3$5	-	-	-	-	-	-	-	-	-	-	❖	-	-	-	-
$S_3$6	-	-	❖	-	-	-	-	❖	-	-	-	-	-	-	❖
$C_4$1	-	-	-	-	-	❖	-	-	-	-	-	-	-	-	-
$C_4$2	-	-	❖	-	-	❖	-	-	-	-	-	-	-	-	-
$C_4$3	-	-	❖	-	-	❖	-	-	-	-	-	-	-	-	-
$C_4$4	-	-	❖	-	-	-	-	-	-	-	-	-	-	-	-
$C_4$5	-	-	❖	-	-	-	-	-	-	-	-	-	-	-	-
$C_4$6	-	-	❖	-	-	-	-	-	-	-	-	-	-	❖	-

❖: detection and amplification of *T. gondii* DNA in PBM cells by the B1 PCR

-: no detection of *T. gondii* DNA in PBM cells by the B1 PCR

6.3.2 Humoral immune response

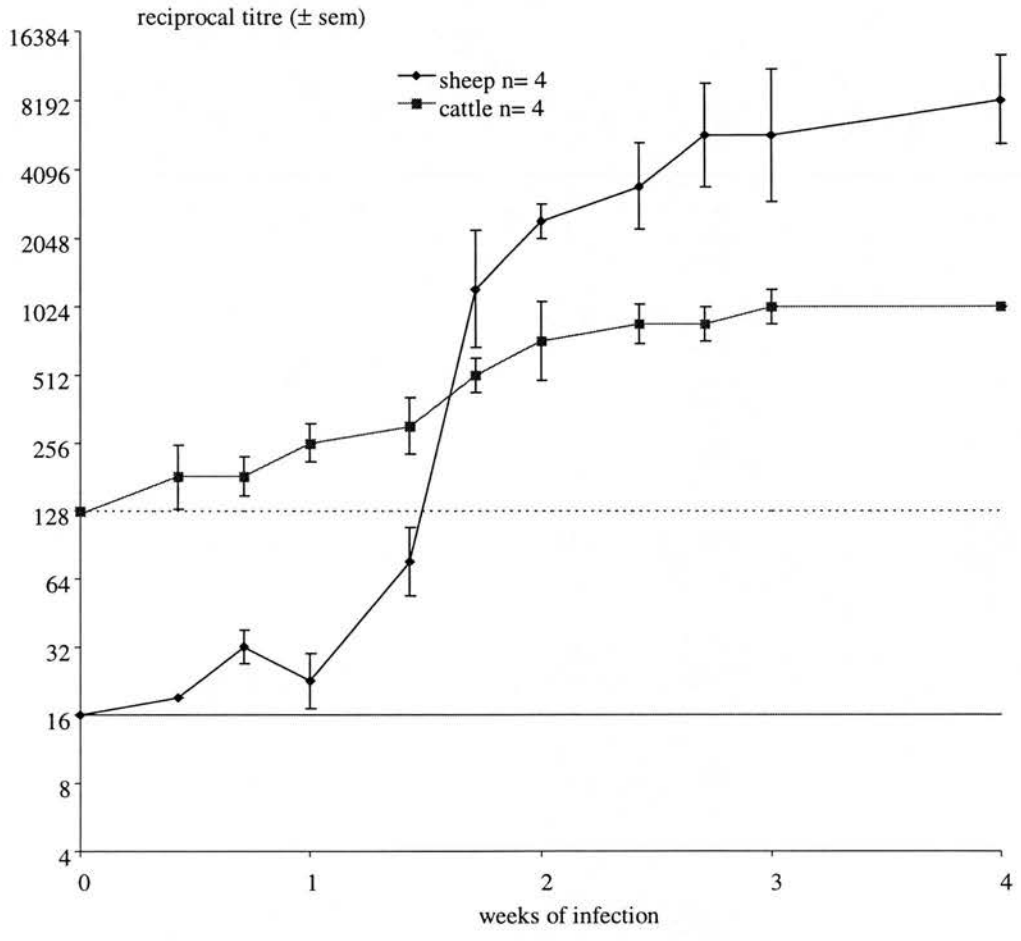
The mean titres of IgG antibody obtained by IFAT are shown in Figure 6. 2 to Figure 6. 5. All the sheep and cattle orally dosed with either 10^3 or 10^5 *T. gondii* sporulated oocysts of the M3 isolate seroconverted after infection. The base line values are a mean of the IgG titres of all the animals in each group before infection. Four samples were collected before infection.

6.3.2.1 Primary infection of sheep and cattle with 10^3 T. gondii oocysts

The IgG IFAT titres for sheep and cattle infected with 10^3 oocysts for a period of four weeks following infection are shown in Figure 6. 2. Neither the sheep nor the cattle seroconverted during the first week after infection with 10^3 oocysts. During the second week the mean titre rose from 1/32 to 1/2048 in the sheep, while the cattle mean antibody titre rose from 1/256 to 1/512. The latter reached a maximum mean IFAT titre by the third week while the sheep reached a maximum mean titre at the fourth week following infection.

The sheep showed a nine-fold increase in antibody titre following infection compared with a three-fold increase in the cattle.

Figure 6. 2 Mean (± sem) *Toxoplasma* IgG IFAT titres in sheep and cattle following primary oral infection with 10^3 oocysts of the M3 isolate



The base line value for the sheep group is represented by the continuous line at 1/16 and for the cattle group by a dotted line at 1/128

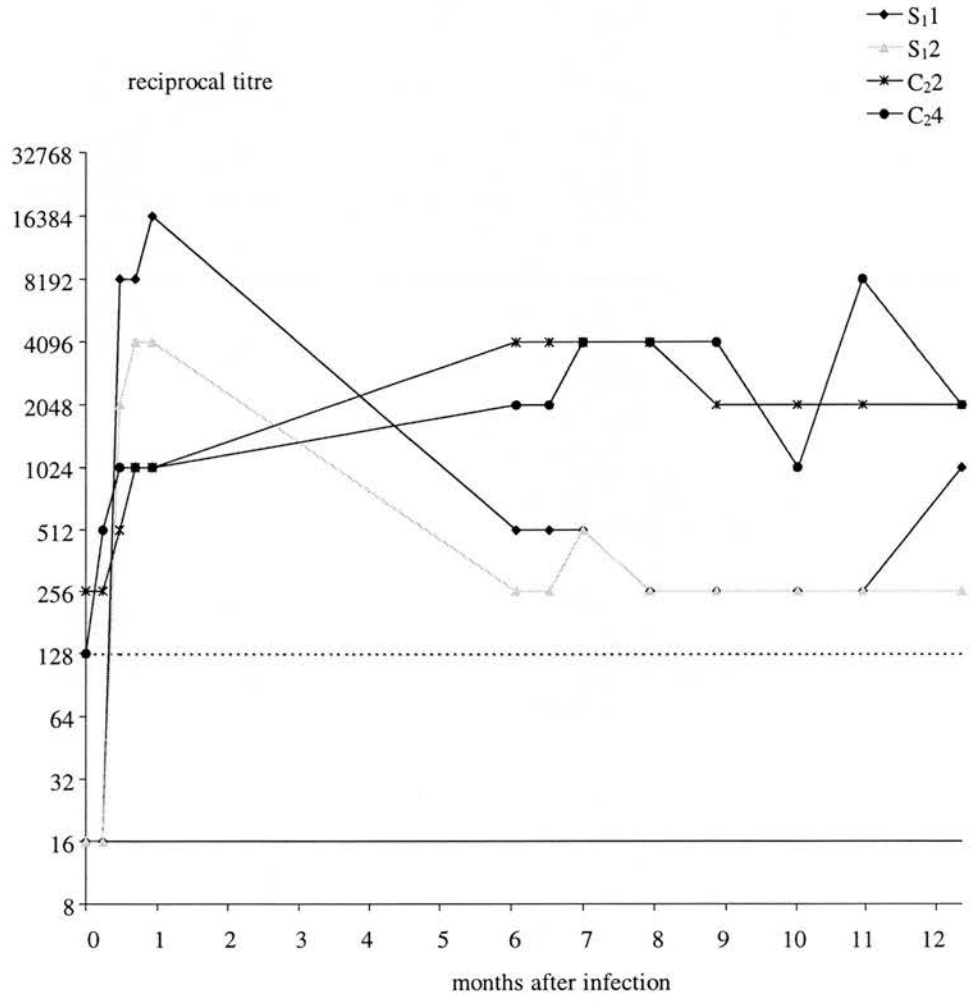
Six weeks following infection, two sheep and two cattle were killed and post mortem examinations carried out. The remaining animals were monitored for specific antibody for 1 year following infection. The IgG IFAT titres of the 2 sheep and 2 cattle sampled every 4 weeks following infection for a period up to 1 year are shown in Figure 6. 3.

The sheep reached a mean maximum titre ($S_1$1:1/16384, $S_1$2: 1/4096) by the end of the first month, after which titres started to decline until six months after infection. Thereafter titres stabilized at 1/256-1/512 (Figure 6. 3).

The two cattle showed a peak IFAT titre of 1/4096 around 6 months after infection. The titre fluctuated slightly around this value during the second six month period.

In general the antibody titres remained fairly stable in both the sheep and cattle from around 6 months after infection, although the antibody titres of the cattle were greater than those of the sheep.

Figure 6. 3 *Toxoplasma* IgG IFAT titres in 2 sheep and 2 cattle each orally dosed with 10^3 oocysts of M3 isolate



The base line value for the sheep is represented by the continuous line at 1/16 and for the cattle by a dotted line at 1/128.

6.3.2.2 Primary infection of sheep and cattle with 10^5 **T. gondii** *oocysts*

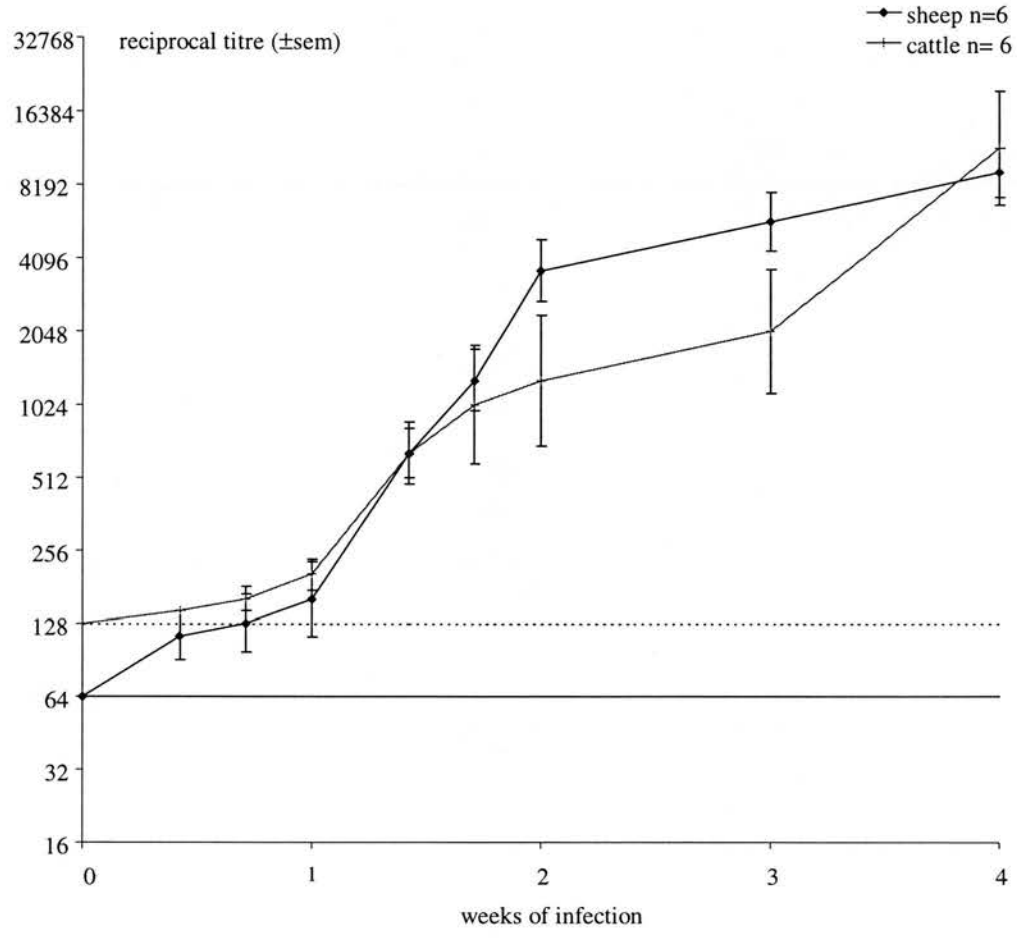
The IgG IFAT titres for sheep and cattle infected with 10^5 oocysts for a period of four weeks and six months following infection are shown in Figure 6. 4 and Figure 6. 5 respectively. The base line values are a mean of the IgG titres of all the animals in each group before infection. Four samples were taken before infection.

Specific antibody was detected in both sheep and cattle following infection. A rapid rise in the IFAT titres was observed in both groups during the second week after dosing and both groups reached maximum titres (1/16384) by the end of the fourth week (Figure 6. 4).

Three sheep and 3 cattle were killed and a postmortem examination conducted at six weeks after infection. Three sheep and 3 cattle were monitored for a period of six months following infection (Figure 6. 5). Both sheep and cattle reached maximum mean antibody values (1/16384 and 1/8192 respectively) within the second month following infection, and titres then started to decline until the fourth month after infection. Thereafter titres stabilized for the remainder of the experiment (sheep: 1/4096-1/8192, cattle: 1/2048-1/4096).

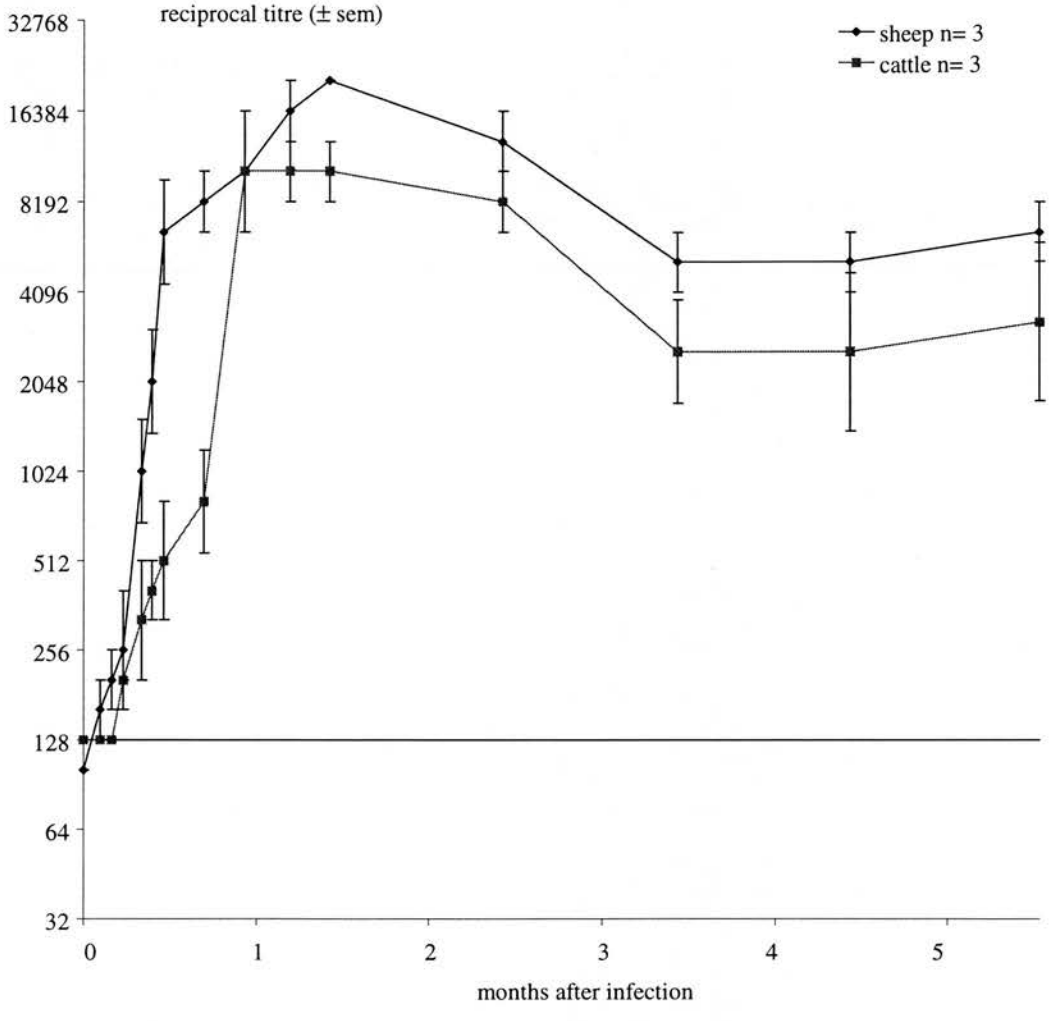
In general the pattern of IFAT responses in sheep and cattle following infection with 10^5 oocysts was very similar.

Figure 6. 4 Mean (± sem) *Toxoplasma* IgG IFAT titres in sheep and cattle following primary oral infection with 10^5 oocysts of the M3 isolate



The base line value for the sheep is represented by the continuous line at 1/64 and for the cattle by a dotted line at 1/128

Figure 6. 5 Mean (± sem) *Toxoplasma* IgG IFAT titres in 3 sheep and 3 cattle following primary oral infection with 10^5 oocyst of the M3 isolate



The base line value for the sheep and cattle is represented by the continuous line at 1/128

6.4 Discussion

The aims of this study were firstly to compare duration and severity of the temperature response, parasitaemia and other clinical signs in sheep and cattle following primary oral infection with either 10^3 or 10^5 *T. gondii* oocysts and secondly to compare IgG IFAT titres in the two groups.

In general in normal individuals, infection with *Toxoplasma* provokes a mild febrile response, accompanied by moderate non-specific symptoms, often remaining unnoticed in natural infection (Costa *et al.*, 1977; Munday, 1978; Blewett *et al.*, 1982; Miller *et al.*, 1982; Dubey, 1983, 1984; Buxton *et al.*, 1988). A more detailed examination of clinical responses is possible when carrying out experimental infections with animals.

After oral infection of sheep and cattle with either a low (10^3) or a high (10^5) dose of *T. gondii* oocysts, it was shown that sheep developed an earlier febrile response than cattle and the time of onset of the pyrexia was dependent on the dose given. Furthermore, the pyrexia lasted longer in the sheep than in the cattle. There was only sporadic detection of circulating *T. gondii* DNA in sheep and cattle and it was more frequently detected in animals dosed with 10^5 oocysts than with 10^3. Both doses of *T. gondii* oocysts induced seroconversion in sheep and cattle. A nine-fold increase in antibody titre was recorded in the sheep compared with a three-fold increase in the cattle following infection with 10^3 oocysts. Whereas the patterns of IFAT responses in sheep and cattle following infection with 10^5 oocysts were more similar. Thus it is concluded that infection was established in all sheep and cattle.

A febrile response following experimental *T. gondii* infection is a common feature in most experimentally infected animals. There are comparatively few reports in the literature concerning clinical aspects of *T. gondii* infection in cattle. Costa and collaborators (1977) described an elevation of the body temperature in calves dosed orally with sporulated *T. gondii*

oocysts (10^4, 10^5 or 3.5×10^5). A rise in temperature was first detected around day two after infection and lasted until day fifteen. The duration of the temperature response was longer than in the experiment reported here, however the authors only monitored one animal per dose of oocysts.

In another report of the experimental infection of cattle with *T. gondii* (Munday, 1978) the onset of fever occurred at a similar time to the study reported in this chapter.

Regardless of whether animals are dosed with *T. gondii* oocysts (Buxton *et al.*, 1988), tissue cysts (Miller *et al.*, 1982) or tachyzoites (Buxton *et al.*, 1991) a temperature response will be elicited.

Buxton and co-workers (1988) reported no significant difference in the onset or duration of the febrile response following oral infection of sheep with two different doses of *T. gondii* oocysts. The main difference between their experiment and the one reported in this chapter is that the oocyst dose ranged from 2×10^3 to 12×10^3 in the former (Buxton *et al.*, 1988) whereas in this chapter the range of the dose administered was greater (10^3 to 10^5). These results together with the findings described in Chapter 4 of this thesis, suggest that the time to onset of a febrile response is dose dependent. The higher the dose of oocysts the earlier the onset of temperature. An additional study reported by Blewett *et al.* (1982) on the febrile response following subcutaneous inoculation with either 75, 250 or 1000 *T. gondii* tissue cysts would agree with the findings from the study reported in this chapter.

It is known that the cytokine IL-1 is an endogenous pyrogen and that prostaglandin E_2 is involved in differentiation of immature B cells as well as suppression of a variety of relevant immune functions (lymphocyte proliferation, cytotoxic T lymphocytes, NK cells and cell to cell interaction). Induction of both immune factors is associated with the quantity of antigen *in vivo* which comes into contact with macrophages (Austyn and Wood, 1993). Thus if a febrile response is elicited earlier in sheep than in cattle, it may reflect

the parasites being present in greater number in the sheep compared to the cattle.

When *Toxoplasma* infection occurs in cattle and sheep, ingested oocysts encyst in the small intestines, each releasing eight sporozoites. Four days later organisms, now defined as tachyzoites, may be present in the mesenteric lymph nodes (Dubey, 1983, 1984). The tachyzoites then migrate to the blood stream and in this way *T. gondii* is disseminated to many tissues (Buxton and Innes, 1995). It has been suggested that the cessation of parasitaemia coincides with the onset of a protective immune response, resulting in the infection persisting in the host as bradyzoites within tissue cysts (Wong and Remington, 1993).

Several studies have described parasitaemia in sheep after infection with *T. gondii*, lasting from the fifth until the twelfth day after infection (Dubey and Sharma, 1980; Reid *et al.*, 1982; Wastling *et al.*, 1993). Jacobs and Hartley (1964) as well as Dubey and Sharma (1980) have described a transient parasitaemia in sheep. Whereas in other hosts, such as goats, rabbits, mice and pigeons, parasitaemias of longer duration have been observed (Jacobs and Jones, 1950; Jacobs *et al.*, 1953; Dubey *et al.*, 1980).

Success in detecting parasitaemia in experimentally infected cattle has been variable. In a study reported by Dubey (1983) where 16 calves and 6 cows were infected with 10^5 oocysts, no parasitaemia was detected. In the study reported here cattle infected with 10^5 oocysts did show a transient parasitaemia. While the detection method used by Dubey (1983) was the inoculation of mice, the B1 PCR technique was used in the study reported here. However studies by Wastling and collaborators (1993) and Buxton and co-workers (1994) demonstrated the very close correlation of the two techniques. It is important to mention that whilst the mouse inoculation technique detects the presence of viable parasites, the PCR would not be able to distinguish whether the parasite DNA detected belongs to a viable organism.

In 1966 Rommel and co-workers (cited by Dubey, 1986b) detected parasite in only 2 of 13 blood samples from cattle experimentally infected with

Toxoplasma tissue cysts (on 2 days in one calf and 1 day in the other) (the dose was not cited by Dubey, 1986b). Costa and collaborators (1977) also reported the detection of *T. gondii* in the blood of 3 out of 3 calves orally infected with 10^4, 10^5 or 3.5×10^5 *T. gondii* oocysts, and in 2 out of 2 calves infected either subcutaneously or orally, with 10^3 *T. gondii* tissue cysts. Parasite was demonstrated as early as six days and as late as sixty-two days after infection . On the contrary, Dubey (1983) described the lack of detectable parasite in the blood of cattle orally dosed with 10^5 oocysts.

The results of the experiments presented in this chapter concur with those of Rommel and collaborators (cited by Dubey, 1986b) and Costa and collaborators (1977) in which the parasite was detected in the blood of cattle. However, it is noticeable that parasite DNA was detected on only a few occasions in animals dosed with the lower number of oocysts (10^3) but that DNA was detected more frequently when the dose was increased to 10^5 oocyst per animal. It is also interesting to note that parasite DNA was found as early as forty-eight hours after infection in cattle dosed with 10^5 oocysts.

Evidence of the parasite in the blood was sporadic apart from the cattle given 10^5 oocysts where 5 out of 6 animals were positive 48 hours after infection. Previous studies employing the PCR, showed that detection of parasite DNA after experimental infection of other animal species was also sporadic. Joss and co-workers (1993) detected *Toxoplasma* DNA (Beverley strain) in blood from Cotton rats and mice by means of B1 PCR from days 2 to 32 after intraperitoneal inoculation and Miedouge (1994) (cited by Paugam *et al.*, 1995) detected *Toxoplasma* (Beverley strain) in blood 7 to 28 days after intraperitoneal inoculation in mice by PCR. Hitt and collaborators, (1992) detected *Toxoplasma* DNA (C56 strain) by B1 PCR in blood of rabbits from days 6 to 32 after subcutaneous inoculation. Schoondermark and collaborators, (1993) detected *Toxoplasma* DNA (RH strain) by means of ribosomal PCR in blood of pregnant monkeys for about 10 days after intravenous inoculation and

Wastling and collaborators (1993) revealed specific DNA in the blood of sheep subcutaneously injected with 10^5 *T. gondii* tachyzoites of the S48 strain.

In the study reported here DNA specific to *T. gondii* was detected as late as 14 days after infection in one case but since the sampling period did not extend beyond this time, it is not possible to say whether or not the parasite would normally circulate later than this.

The apparent sporadic nature of the parasitaemia may be because animals were sampled only once per day. A further experiment in which several samples are collected each day might reveal a more accurate picture of when *T. gondii* is present in blood following infection.

One of the aims of this chapter was to compare the onset, duration and magnitude of the IgG antibody response in sheep and cattle experimentally infected with *T. gondii*.

Although the sheep and cattle were initially infected with the same dose of oocysts, either 10^3 or 10^5, both innate and acquired immune mechanisms may be involved in affecting parasite multiplication *in vivo* and hence the real dose of infection within the sheep and cattle may differ. It is known that antibody production is driven by the quantity of antigen *in vivo* and that as the concentration of antigen decreases so production of antibody will also decrease. (Roitt, 1991). Therefore a difference between the magnitude of the antibody response between sheep and cattle may reflect differences in the actual quantity of parasites *in vivo*. Thus if cattle are more efficient at controlling *T. gondii* multiplication early on *in vivo* compared with sheep, then it might be predicted that this may be reflected in the subsequent magnitude of the antibody response. This did appear to be the case when comparing groups of sheep and cattle infected with 10^3 *T. gondii* oocysts, where the former showed a nine-fold increase in antibody titre whereas the cattle only had a three-fold increase. A similar trend, although not so marked, was observed when 10^5 oocysts were given. These data may provide indirect evidence that cattle were better able to control the multiplication of *T. gondii in vivo* compared to the sheep.

The study in cattle reported by Costa and collaborators (1977) suggested that the higher the infective dose of *T. gondii* the larger the subsequent antibody response. The data from this study would agree with this as cattle with 10^3 *T. gondii* oocysts developed a lower specific IgG response than the cattle receiving 10^5 *T. gondii* oocysts. This relationship between infective dose and antibody titre was not so obvious in the group of sheep.

The maintenance of antibody titres over time is thought to reflect persistence of antigen within the host. Where antigen does not persist antibody levels decline. This has been demonstrated quite elegantly in experiments carried out in sheep infected with the S48 strain of *T. gondii*, which is an incomplete strain of the parasite and is not thought to persist (Wilkins *et al.*, 1987). In these animals specific antibody levels declined 30 weeks after the initial infection (Buxton *et al.*, 1993b).

Cattle are thought to harbour fewer *T. gondii* cysts in their tissues than sheep (Dubey, 1986b) and these cysts may not persist in cattle as they do in sheep. Therefore measurement of antibody levels over time may reflect whether *T. gondii* was persisting within the animals. In this study cattle and sheep were monitored for up to 1 year following infection with 10^3 *T. gondii* oocysts. In both cases specific antibody was detected over the time period suggesting that *T. gondii* infection persisted within both sheep and cattle.

In general the cattle had higher IgG IFAT titres prior to infection than the sheep. As so little is understood about *T. gondii* infection in cattle, it was difficult to determine with confidence a cut off titre in the IFAT test which would allow one to distinguish a naive from a previously exposed animal.

There has been no data published to the author's knowledge on secondary *T. gondii* infection of cattle, however there are reports describing the events following secondary infection of sheep. The majority of the reports concur that on a secondary infection with *T. gondii*, regardless of the stage of the parasite used, there is no boost to antibody levels and no pyrexia is provoked (Blewett *et al.*, 1982, 1983; McColgan *et al.*, 1988; Buxton *et al.*,

1991; Innes and Wastling, 1995). As the cattle in this study showed a significant febrile response and increase in specific antibody titre, it is concluded that they were undergoing primary infection.

6.5 Conclusions

The sheep developed an earlier temperature response which lasted longer than the cattle and they also showed a greater magnitude of antibody response compared to cattle. These findings taken together suggest that the cattle were better able to control early multiplication of *T. gondii* better than sheep.

However the innate and specific immune mechanisms responsible for the enhanced ability of cattle to control the parasitaemia are unknown.

Cell mediated immune mechanisms are thought to be important in resolving primary *T. gondii* infection (Frenkel, 1967; Krahenbuhl and Remington, 1982; Suzuki *et al.*, 1988; Suzuki and Remington, 1988; Nagasawa *et al.*, 1991; Gazzinelli *et al.*, 1991). The following chapter examines aspects of cell mediated immune response in sheep and cattle following infection with *T. gondii*.

Chapter 7

Cellular immune response of sheep and cattle to *Toxoplasma gondii* infection

Aims

To measure cell mediated immune responses in cattle and sheep during a primary infection with *T. gondii* induced by the oral administration of oocysts.

7. Cellular immune responses of sheep and cattle to *Toxoplasma gondii* infection

7.1 Introduction

As *T. gondii* is an intracellular parasite, cell-mediated immunity would be expected to play a major role in host defence. By far the majority of data related to immune mechanisms have originated from studies using experimentally infected mice. While providing important information on the mechanisms involved in the control of *Toxoplasma* infection, attention should be paid when attempting to extrapolate data to other animal species due to the wide variation in vulnerability to *T. gondii* infection between different animal species.

Studies using *T. gondii* infected mice have demonstrated the importance of IFNγ in protective immunity (Subauste and Remington, 1991). This cytokine is produced by both $CD4^+$ and $CD8^+$ T cells with more recent data demonstrating the role of natural killer (NK) cells in the early production of IFNγ (Gazzinelli *et al.*, 1993). Adoptive transfer experiments between inbred mouse strains have demonstrated the importance of $CD8^+$ T cells in controlling a primary infection (Suzuki and Remington, 1988). While it may be possible to generate $CD8^+$ effector cells without $CD4^+$ help, optimal activation is achieved when the two T cell subsets act in synergy. The $CD4^+$ T cells (predominantly the Th1 subtype) are known to produce the cytokine IFNγ which is thought to be necessary in the induction of a $CD8^+$ response. In one study in which $CD4^+$ T cells were depleted from mice, inhibition of the $CD8^+$ T cell response was seen. The inhibition could be partially corrected by the addition of recombinant IL-2, suggesting that the generation of cytotoxic $CD8^+$ effector cells was

enhanced by IL-2 production from $CD4^+$ T cells (Leist *et al.*, 1989). Innes and collaborators (1995a) reported that $CD4^+$ T lymphoblasts preceded the appearance of $CD8^+$ T lymphoblasts in responding lymph to *T. gondii* infection and the addition of exogenous IL-2 was a necessary requirement to activate $CD8^+$ T cells *in vitro*.

There are limited studies on the immune response to *Toxoplasma* infection in other animal species. In sheep, the incomplete S48 strain (Wilkins *et al.*, 1987) of *T. gondii* which lacks the ability to produce tissue cysts (and therefore to establish persistent infection) has been used in experimental infections. Following subcutaneous inoculation of naive sheep with S48 tachyzoites, phenotypic analysis of lymphoblast cells in the efferent lymph showed that the cells responding to infection were mainly T cells. Initially those with a $CD4^+$ phenotype were more numerous than CD8+ cells but at around day 9 to 10 after infection, at the time of peak lymphoblast response, the $CD8^+$ cells became predominant. The disappearance of the parasite from the lymph also occurred at this time (Innes and Wastling, 1995). IFNγ was detected in cell-free lymph supernatant from days 3 to 10 after primary infection but appeared in lymph as early as 24 hours after secondary challenge (Innes *et al.*, 1995b). Both $CD4^+$ and $CD8^+$ T cell subsets have the potential to produce IFNγ after appropriate stimulation (Morris *et al.*, 1982; Mosmann and Coffman, 1987). IFNγ production, originated predominantly from the $CD4^+$ T cell subset was demonstrated after stimulation of immune spleen cells *in vitro* with a crude tachyzoite antigen (Gazzinelli *et al.*, 1991). Similar results were described by Innes and collaborators (1995a, b) after examining the *in vivo* immune response of sheep to subcutaneous (s.c.) inoculation of S48 strain *T. gondii* tachyzoites. These observations suggest that a T cell response combined with IFNγ production plays a major role in immunity to *T. gondii* infection in sheep. These *in vivo* observations are further supported by functional *in vitro* studies which have shown that recombinant ovine IFNγ will inhibit the growth of *T. gondii* in ovine cells (Oura *et al.*, 1993).

In an attempt to understand the role of cellular immune mechanisms in the innate resistance of cattle to *T. gondii* infection, experiments were set up to monitor antigen specific lymphocyte proliferation responses, the presence of IFNγ in blood plasma and in supernatants from activated cells, and an analysis of the T cell subsets in peripheral blood of cattle and sheep during a primary oral infection with *T. gondii* oocysts.

7.2 Materials and methods

7.2.1 Animals

Ten adult sheep (ages ranging from 2 to 5 years) and ten calves (ages ranging from 6 to 7 months) shown to be seronegative by IFAT to *T. gondii* at the start of the experiment were used in this study.

Mature Swiss White mice of both sexes from *Toxoplasma* free colonies were used to passage and maintain the S48 strain of *T. gondii* tachyzoites as described in Chapter 3 (3.1.3).

Cats of both sexes, under six months of age and seronegative by IFAT for antibodies to *T. gondii*, obtained from a *Toxoplasma* free colony in the University of Newcastle (UK), were used in this study.

7.2.2 *Toxoplasma gondii*

Oocysts were prepared from the faeces of seronegative cats which had been fed *T. gondii* tissue cysts as described in Chapter 3 (3.2.1.3).

A crude sonicated lysate of *T. gondii* tachyzoites prepared as described in the Chapter 3 (3.3) was used as the specific antigen in the proliferation assay.

7.2.3 Experimental design

Sheep and cattle were allocated into four groups as described in Table 7. 1. Doses of either 10^3 or 10^5 *T. gondii* oocysts were administrated by gavage. These are the same experimental groups used in Chapter 5 of this thesis.

Table 7. 1 Experimental design

Group	Species	Animal numbers	M3 oocysts
1	sheep	$S_1$1, $S_1$2, $S_1$3, $S_1$4	10^3
2	cattle	$C_2$1, $C_2$2, $C_2$3, $C_2$4	10^3
3	sheep	$S_3$1, $S_3$2, $S_3$3, $S_3$4, $S_3$5, $S_3$6	10^5
4	cattle	$C_4$1, $C_4$2, $C_4$3, $C_4$4, $C_4$5, $C_4$6	10^5

S_n**N**: sheep number **N** from group **n**, C_n**N**: cattle number **N** from group **n**

7.2.4 Cellular immune response

Blood samples from sheep and cattle were collected from the jugular vein into preservative-free heparinised evacuated tubes. Cell free peripheral blood plasma was separated by centrifugation and PBM cells were separated by centrifugation over Lymphoprep (Nycomed, Norway) (Chapter 3 3.4.2, 3.4.3).

7.2.4.1 Phenotypic analysis of peripheral blood mononuclear cells

An indirect immunofluorescence test was used to stain the peripheral lymphocytes from the sheep and cattle infected with 10^5 oocysts as described in Chapter 3 (3.7.2). Three base line samples were collected prior to infection and thereafter samples were collected every second day for three weeks.

7.2.4.2 Cytokine analysis

Cell free peripheral blood plasma and supernatants from short-term cultured PBM cells were tested for the presence of biologically active IFNγ using a bioassay described by Entrican and collaborators (1989, 1992) and an enzyme immunoassay test (CSL Veterinary, Australia) as described by the manufacturer. These methods are described in full in Chapter 3 (3.7.3).

7.2.4.3 Antigen induced proliferation assay on peripheral blood mononuclear cells

A proliferation assay on peripheral blood mononuclear cells was carried out to monitor the response of cells to the crude lysate antigen of *T. gondii* antigen as described by Innes and collaborators (1995a) (see Chapter 3, 3.7.1).

In brief, 4×10^5 cells were cultured in each well of a 96 well round bottomed plate along with a known dilution of *T. gondii* antigen. Concanavalin A was used as positive control, to demonstrate that the cells were able to respond in the culture conditions of the test and medium alone without antigen as a negative control to examine the spontaneous proliferation of cells in the absence of specific antigen. The cells were cultured for five days at 37°C in a humidified 5% CO_2 incubator, they were pulsed for the final 18 hours with 18.5 kBq [^{3}H]-thymidine per well prior to harvesting onto filters and cell associated radioactivity was quantified in a β Scintillation counter.

7.3 Results

7.3.1 Phenotypic analysis of peripheral blood mononuclear cells

An indirect immunofluorescence test was used to stain the PBM cells from sheep and cattle undergoing primary oral infection with *T. gondii*, using the appropriate monoclonal antibodies at a pre-determined optimal dilution.

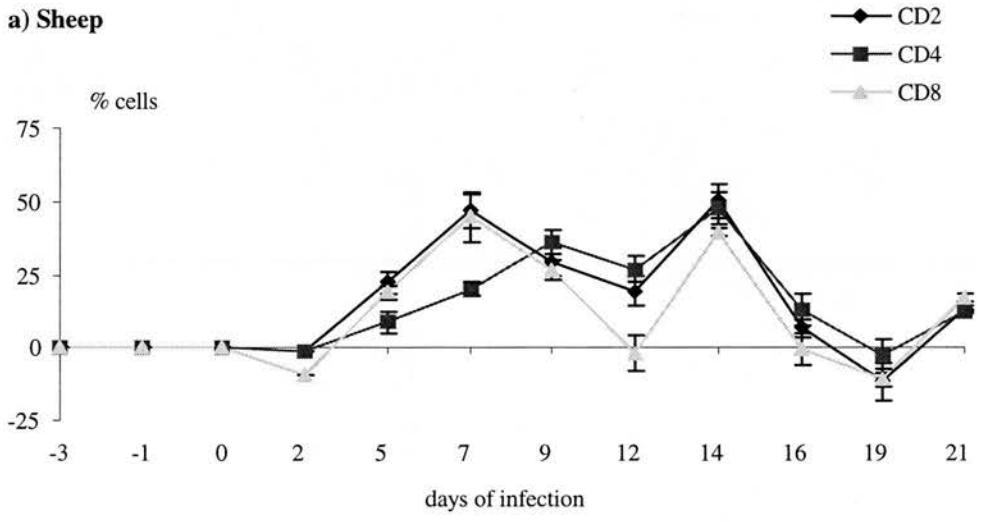
Figure 7. 1 and Figure 7. 2 illustrate the mean percentage change from base line values of $CD2^+$, $CD4^+$ and $CD8^+$ T cells present in peripheral blood of six sheep and six cattle, both before and after infection with 10^5 oocysts. Data showing the percentage composition of peripheral blood mononuclear cells for individual animals are shown in Appendix Chapter 7 (Table 1 to Table 6).

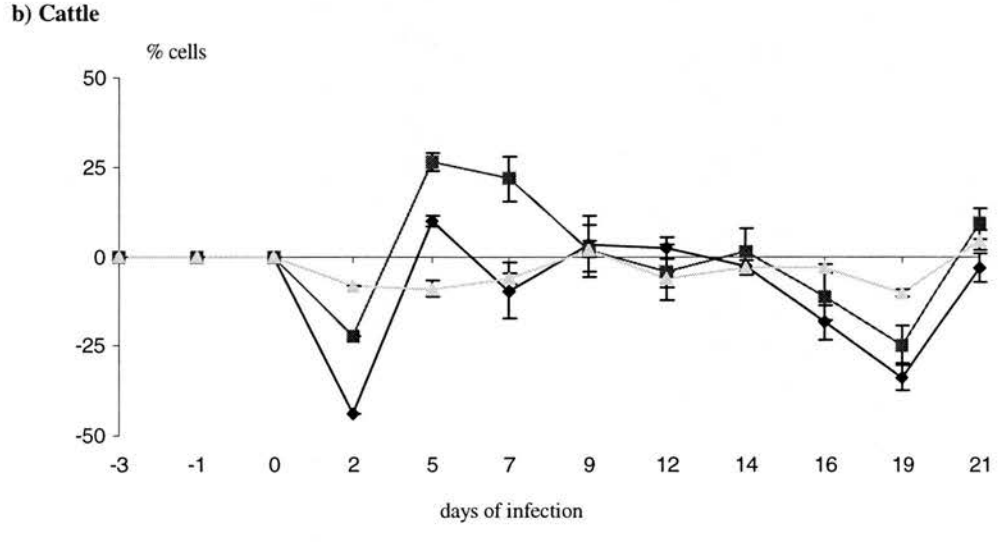
The three base line values for each T cell subset were calculated as the mean percentage of each phenotype before infection. The percentage change of sheep $CD2^+$ T cells increased from pre-infection base line values by day five after infection (Figure 7.1a). The percentage of these cells increased steadily during the experimental period, reaching maximum values on days 7 and 14, and declined back to base line levels around day 16-19 post infection.

Study of the response of the two main peripheral T cell subsets from sheep showed a significant increase in the percentage of $CD4^+$ cells from day five after infection and showed maximum values on day 14 (Figure 7.1a). The percentage of cells stained with the monoclonal antibody recognising $CD8^+$ T cell subset declined briefly during the first 48 hours after infection then rose to exceed pre-infection base line values from day five.

The results in Figure 7.1b show the mean results from of all the cattle orally infected with 10^5 oocyst of *T. gondii*. The numbers of $CD2^+$, $CD4^+$ and $CD8^+$ T cells declined briefly during the first 48 hours of infection. During the monitored period of 21 days of infection, only the percentage of $CD4^+$ T cells increased from pre-infection base line values from day five after infection reaching the maximum value, after which they declined again to values below base line on day nine.

Figure 7. 1 Mean (n=6) percentage change from base line values (± sem) of ovine and bovine peripheral blood lymphocyte cells staining with monoclonal antibodies recognizing different lymphocyte subsets following a primary oral infection with 10^5 *T. gondii* oocysts





n= group size

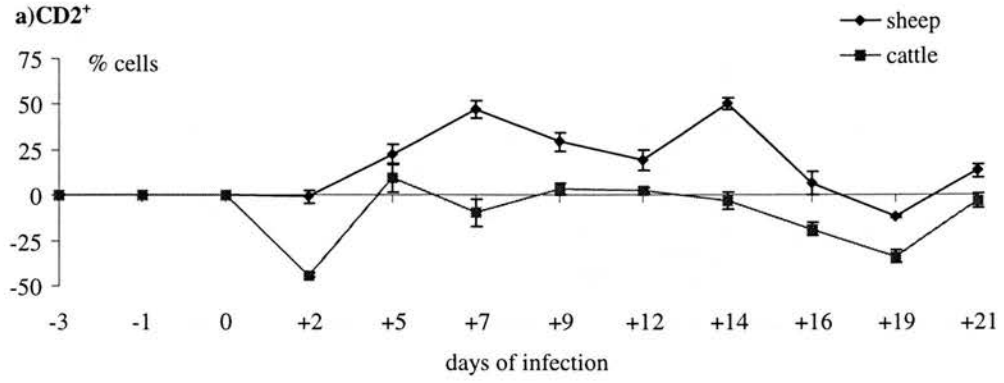
Figure 7. 2 illustrates the mean change in percentage (relative to base line values) of sheep and cattle peripheral blood T cells staining with monoclonal antibodies recognizing $CD2^+$, $CD4^+$ and $CD8^+$ subsets following a primary oral infection with *T. gondii*.

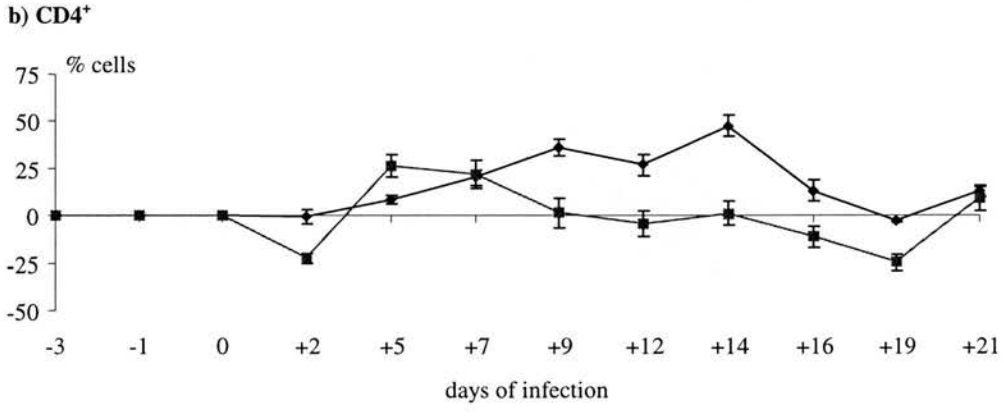
The number of $CD2^+$ T cells increased in sheep from day 5 of infection to a maximal 50% higher than preinfection base line values. In contrast the percentage of $CD2^+$ T cells in cattle showed an initial decrease in the percentage of cell from this subset during the first 48 hours and then values did not change much from base line values (Figure 7.2a).

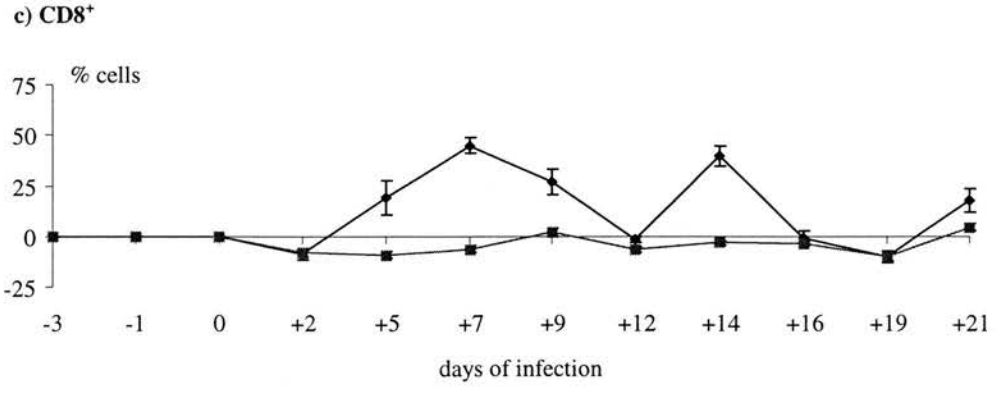
Analysis of $CD4^+$ T cells showed a large increase in the number in the sheep peripheral blood from day seven after infection. In the group of cattle there was a brief increase in the number of $CD4^+$ T cells, which then settled down to base line values on day 9 after infection (Figure 7.2b).

Finally, the percentage of $CD8^+$ T cells within the sheep PBM sample increased, from base line values on day 5 post infection and did not return to preinfection levels until day 16. On the contrary the % of cattle $CD8^+$ T cells in PBM did not change much over the observed time period (Figure 7.2c).

Figure 7. 2 Mean (n=6) percentage change from base line values (± sem) of ovine and bovine peripheral blood $CD2^+$ (a), $CD4^+$ (b) and $CD8^+$ (c) lymphocyte subsets following a primary oral infection with 10^5 *T. gondii* oocysts







7.3.2 Cytokine analysis

Cell free peripheral blood plasma from sheep and cattle undergoing primary oral *Toxoplasma* infection with two different doses of parasite were firstly screened for the presence of IFN using an antiviral bioassay (see Chapter 3, 3.7.3.3). Samples which were positive by bioassay were then analysed specifically for the presence of IFNγ using an ELISA (Chapter 3, 3.7.3.4). Cell free supernatants from short term culture of ovine and bovine PBM cells primed *in vivo* and stimulated *in vitro* with *T. gondii* antigen were collected and analysed for the presence of IFN by the methods described above. Data are represented in Figure 7.3 to Figure 7.9, and Table 7.2 to Table 7.9.

7.3.2.1 Cell free peripheral blood plasma samples

7.3.2.1.1 Oocyst dose: 10^3 *T. gondii* oocysts

The results in Table 7. 2 show biologically active IFN detected in peripheral plasma samples of sheep and cattle undergoing primary oral infection. It can be seen that biologically active IFN was more frequently detected in the group of cattle than the group of sheep. Three out of four sheep were positive in the antiviral bioassay for detection of IFN, while IFN was found in all the cattle. After primary infection with *T. gondii*, biologically active IFN was detectable in ovine cell-free peripheral plasma from day 3 until day 7 of infection. In the cattle group the highest frequencies of positive results were recorded on day 3, 5 and 7 with some outlying positive samples on day 12 to 19 of infection.

Table 7. 2 Biologically active IFN detected in cell free peripheral blood plasma of sheep and cattle orally infected with *T. gondii* oocysts measured by an antiviral bioassay

Oocyst dose: 10^3 *T. gondii* oocysts													
Animal	days of infection												
	-3	-1	2	3	5	7	10	12	14	17	19	21	26
$S_1$1	-	-	-	-	-	❖	-	-	-	-	-	-	-
$S_1$2	-	-	-	❖	-	-	-	-	-	-	-	-	-
$S_1$3	-	-	-	❖	❖	-	-	-	-	-	-	-	-
$S_1$4	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_2$1	-	-	-	❖	❖	❖	-	❖	-	❖	-	-	-
$C_2$2	-	-	-	❖	❖	❖	-	-	❖	-	-	-	-
$C_2$3	-	-	-	❖	❖	❖	-	-	-	-	❖	-	-
$C_2$4	-	-	-	-	❖	❖	-	-	❖	❖	-	-	-

❖: biologically active IFN detected by an antiviral bioassay

-: no detection of biologically active IFN by an antiviral bioassay

Cell-free peripheral blood plasma samples from sheep and cattle in which biologically active IFN was detected were analysed for the presence of IFNγ using an ELISA. The ELISA was calibrated using standards of recombinant bovine and ovine IFNγ prepared from doubling dilutions of a stock of rIFNγ and the values of %OD obtained were graphically represented *versus* the corresponding known amounts of IFNγ for each %OD and expressed in U/ml for sheep (Figure 7. 3) and in pg/ml for cattle (Figure 7. 4).

One unit of IFN corresponds to the lowest concentration of recombinant (r) IFNγ which would inhibit the cytopathic effect of SFV on target cells by greater than 50% (Entrican *et al.*, 1989, 1992) and that approximately 50pg/ml of bovine rIFNγ correspond to 1U/ml (personal communication by Dr. G. Entrican). Summary of the values of ovine and bovine rIFNγ standards are displayed in Table 7. 3.

Figure 7. 3 Ovine recombinant IFNγ standards analysed by ELISA

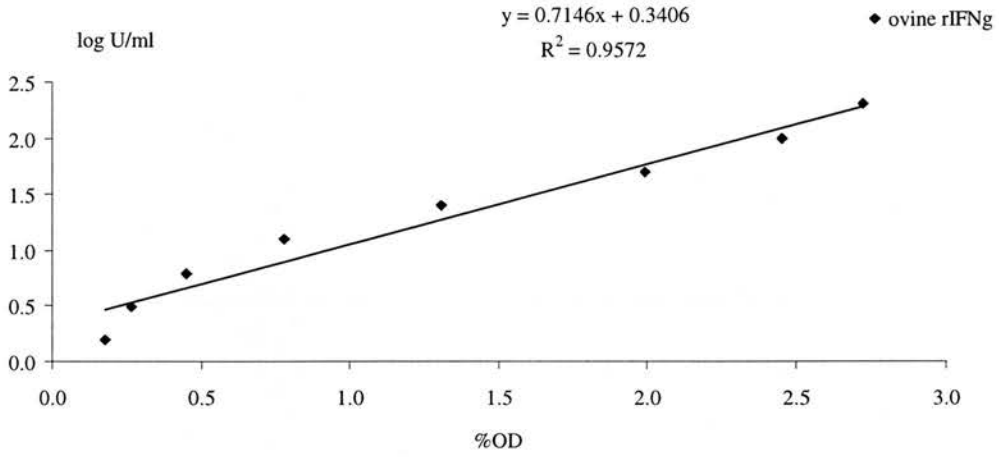


Figure 7. 4 Bovine recombinant IFNγ standards analysed by ELISA

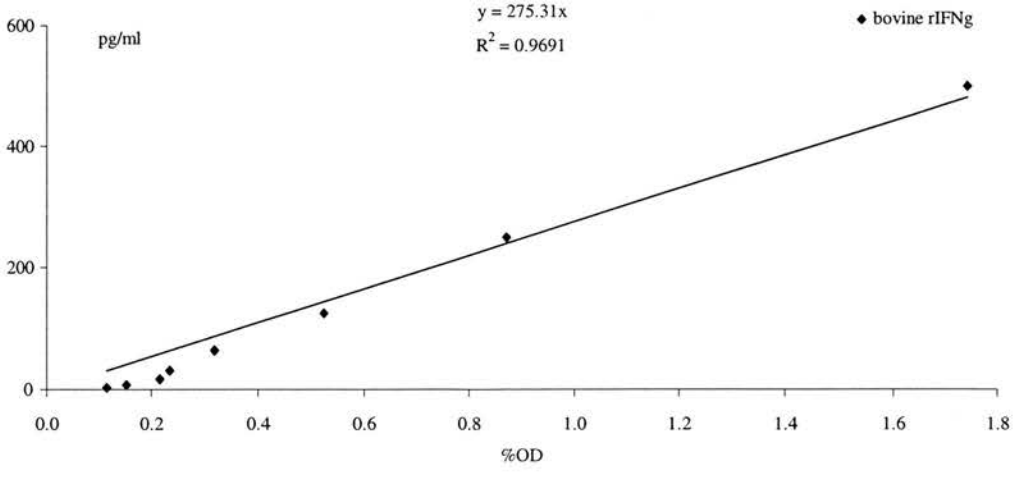


Table 7. 3 Ovine and bovine rIFNγ standards using ELISA

bovine standards			ovine standards	
%OD	pg/ml	U/ml	%OD	U/ml
1.743	500	10	2.721	200
0.872	250	5	2.453	100
0.525	125	2.5	1.995	50
0.318	62.50	1.25	1.306	25
0.235	31.25	0.62	0.778	12.50
0.215	15.62	0.31	0.453	6.25
0.152	7.81	0.15	0.270	3.13
0.114	3.13	0.08	0.178	1.56

The amount of IFNγ detected in cell-free peripheral blood plasma from cattle and sheep were calculated from the standard curves represented in Figure 7. 3 and Figure 7. 4 (Mead *et al.*, 1993). They are displayed in Table 7. 4 and represented in Figure 7. 5 and Figure 7. 6.

It can be seen in Table 7. 4 that IFNγ was detected in all the peripheral blood plasma samples from infected sheep which had being previously positive when analysed for the presence of biologically active IFN. Two of the animals presented levels greater than 2 U/ml and one of the sheep had up 6 U/ml of IFNγ in peripheral blood plasma.

In all the *Toxoplasma*-infected cattle levels of IFNγ above 1U/ml were found on day 5 of infection, on day 7 after infection in 2 of the cattle ($C_2$2 and $C_2$3). 3.73 U/ml of IFNγ were detected in plasma sample of one of the cattle ($C_2$4) on day 14 after oral infection (Table 7. 4).

Table 7. 4 IFNγ detected in cell free peripheral blood plasma of sheep and cattle orally infected with 10^3 *T. gondii* oocysts measured by ELISA.

a)sheep

Oocyst dose: 10^3 *T. gondii* oocysts					
Animal	day of infection	% OD	sd	sem	U/ml
$S_1$1	7	0.62	0.026	0.037	6.02
$S_1$2	3	0.13	0.036	0.051	2.69
$S_1$3	3	0.09	0.003	0.04	2.51
	5	0.18	0.029	0.041	2.95

b)cattle

Oocyst dose: 10^3 *T. gondii* oocysts						
Animal	day of infection	% OD	sd	sem	pg/ml	U/ml
$C_2$1	3	0.123	0.023	0.033	33.73	0.67
	5	0.189	0.009	0.013	52.03	1.04
	7	0.093	0.002	0.003	25.47	0.51
	12	0.151	0.001	0.001	41.57	0.83
	17	0.107	0.001	0.001	29.46	0.59
$C_2$2	3	0.083	0.000	0.001	22.71	0.45
	5	0.437	0.023	0.033	120.31	2.40
	7	0.330	0.003	0.004	90.85	1.87
	14	0.069	0.004	0.006	19.00	0.38
$C_2$3	3	0.118	0.004	0.006	32.35	0.65
	5	0.624	0.016	0.022	171.79	3.44
	7	2.148	0.030	0.043	591.37	11.82
	19	0.059	0.006	0.009	16.24	0.32
$C_2$4	5	0.813	0.124	0.175	223.83	4.48
	7	0.081	0.001	0.002	22.30	0.45
	14	0.676	0.035	0.049	186.11	3.72
	17	0.089	0.005	0.007	24.36	0.49

Figure 7. 5 Amount of IFNγ in plasma from sheep undergoing primary oral infection with 10^3 *T. gondii* oocysts (See Table 7.4a for time of plasma sampling)

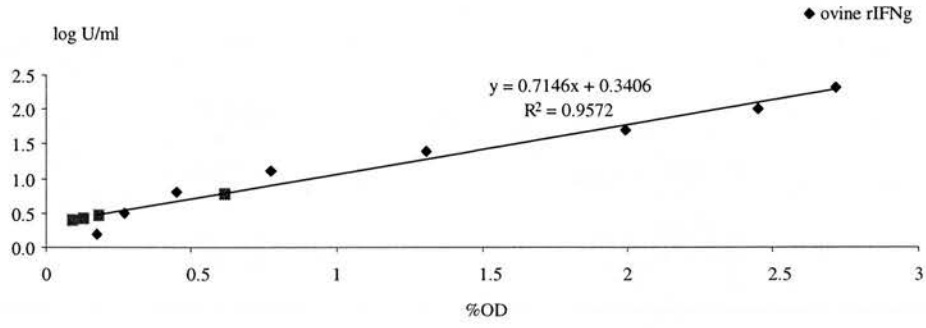
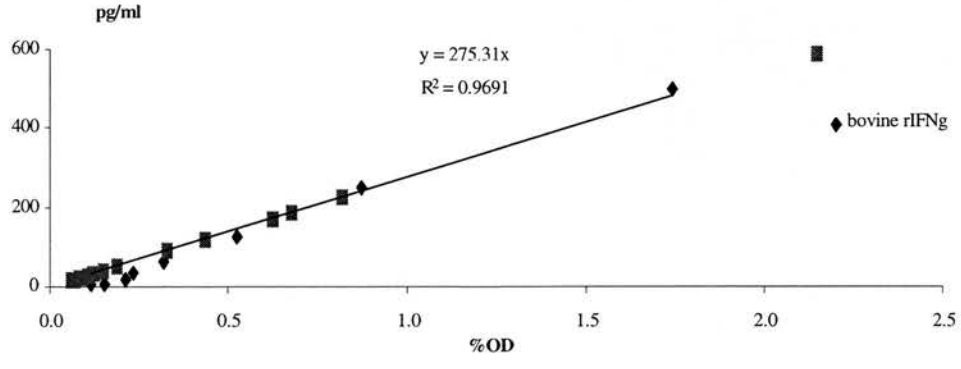


Figure 7. 6 Amount of IFNγ in plasma from cattle undergoing primary oral infection with 10^3 *T. gondii* oocysts (See Table 7.4b for time of plasma sampling)



7.3.2.1.2 Oocyst dose: 10^5 *T. gondii* oocysts

Table 7. 5 displays the results obtained from the antiviral bioassay carried out on peripheral plasma samples of sheep and cattle undergoing primary oral infection with 10^5 *T. gondii* oocysts. IFN. When the oocyst dose was increased from 10^3 (Table 7. 2) to 10^5 (Table 7. 5), biologically active IFN was not detected in the sheep samples, while IFN was detectable in all the cattle between day 2 and 7 after infection.

Table 7. 5 Biologically active IFN detected in cell free peripheral blood plasma of sheep and cattle orally infected with *T. gondii* measured by antiviral bioassay

Oocyst dose: 10^5 *T. gondii* oocysts													
Animal	**days of infection**												
	-3	**-1**	**2**	**3**	**5**	**7**	**10**	**12**	**14**	**17**	**19**	**21**	**26**
$S_3$1	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_3$2	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_3$3	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_3$4	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_3$5	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_3$6	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_4$1	-	-	❖	-	❖	❖	-	-	-	-	-	-	-
$C_4$2	-	-	❖	-	-	❖	-	-	-	-	-	-	-
$C_4$3	-	-	❖	-	-	❖	-	-	-	-	-	-	-
$C_4$4	-	-	❖	-	❖	❖	-	-	-	-	-	-	-
$C_4$5	-	-	❖	-	❖	❖	-	-	-	-	-	-	-
$C_4$6	-	-	❖	-	❖	-	-	-	-	-	-	-	-

❖: biologically active IFN detected by an antiviral bioassay

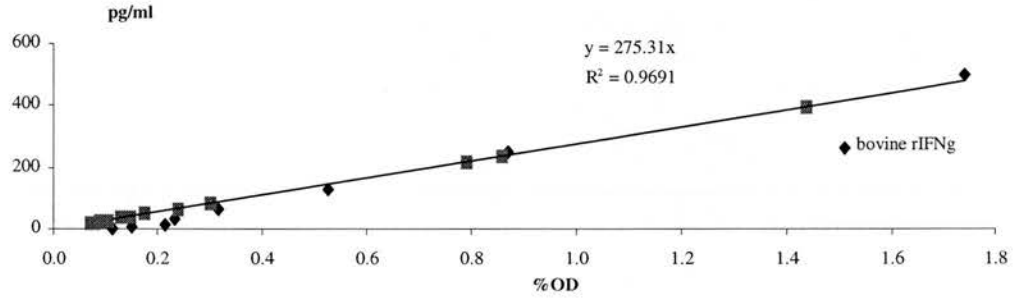
-: no detection of biologically active IFN by an antiviral bioassay

Cell-free peripheral blood plasma samples from cattle in which biologically active IFN was detected were also analysed for the presence of IFNγ by ELISA. Recombinant bovine IFNγ standards were used to determine the amount of IFNγ in cell-free peripheral blood plasma from cattle (Table 7.3 and Figure 7. 4). Data for individual animals are displayed in Table and Figure 7. 7.

Table 7. 6 IFNγ detected in cell free peripheral blood plasma of cattle orally infected with 10^5 *T. gondii* oocysts measured by ELISA.

Oocyst dose: 10^5 *T. gondii* oocysts						
Animal	**day of infection**	**%OD**	**sd**	**sem**	**pg/ml**	**U/ml**
$C_4$1	2	0.070	0.00	0.00	19.27	0.39
	5	0.791	0.03	0.04	217.77	4.36
	7	0.139	0.00	0.00	38.13	0.76
$C_4$2	2	0.076	0.00	0.00	20.92	0.42
	7	0.303	0.09	0.12	83.42	1.67
$C_4$3	2	0.131	0.00	0.00	35.93	0.72
	7	0.076	0.00	0.01	20.92	0.42
$C_4$4	2	1.438	0.12	0.17	395.90	7.92
	5	0.239	0.01	0.01	65.80	1.32
	7	0.091	0.00	0.00	24.92	0.5
$C_4$5	2	0.147	0.00	0.00	40.33	0.81
	5	0.098	0.00	0.01	26.98	0.54
	7	0.102	0.01	0.01	28.08	0.56
$C_4$6	2	0.860	0.03	0.04	236.63	4.73
	5	0.177	0.02	0.03	48.59	0.97
	7	0.104	0.01	0.01	28.63	0.57

Figure 7. 7 Amount of IFNγ in blood plasma from cattle undergoing primary oral infection with 10^5 *T. gondii* oocysts



7.3.2.2 Cell free supernatants from antigen induced proliferation assays on PBM cells

7.3.2.2.1 Oocyst dose: 10^3 *T. gondii* oocysts

Cell free supernatants from short term culture of ovine and bovine PBM cells primed *in vivo* and stimulated *in vitro* with *T. gondii* antigen were collected and analysed for the presence of biologically active IFN using an antiviral bioassay. Data for individual animals are displayed on Table 7. 7.

Biologically active IFN was detected more frequently in supernatants from bovine cells cultured and stimulated with *Toxoplasma* antigen than in sheep cells under the same conditions. IFN was detected in all four sheep infected with 10^3 *Toxoplasma* oocysts, between day 12 and 19 after infection.

In contrast, the presence of biologically active IFN in supernatant from short term cultures of PBMC stimulated with *Toxoplasma* antigen, was shown between days 7 and 26 after infection in all the cattle used in this study. IFN

was detected in 3 out of 4 cattle ($C_2$1, $C_2$2 and $C_2$4) from cell supernatants cultured on day 3 after infection.

There was insufficient supernatant from short term cultures of antigen-stimulated PBM cells from sheep and cattle infected with a low dose of *T. gondii* oocysts (10^3). Therefore, it was not possible to determine if the biologically active IFN detected from the samples mentioned above was IFNγ.

Table 7. 7 Biologically active IFN detected in cell free supernatants from short term culture of PBMC primed *in vivo* after *in vitro* stimulation with *T. gondii* antigen

Oocyst dose: 10^3 *T. gondii* oocysts												
animals	days of infection											
	-2	-1	3	5	7	10	12	14	17	19	21	29
$S_1$1	-	-	-	-	-	-	❖	-	-	❖	-	-
$S_1$2	-	-	-	-	-	-	❖	-	❖	❖	-	-
$S_1$3	-	-	-	-	-	-	-	❖	-	❖	-	-
$S_1$4	-	-	-	-	-	-	-	-	-	❖	-	-
$C_2$1	-	-	❖	-	❖	❖	❖	❖	❖	❖	❖	❖
$C_2$2	-	-	❖	-	❖	❖	❖	❖	❖	❖	❖	❖
$C_2$3	-	-	-	-	❖	❖	❖	❖	❖	❖	❖	❖
$C_2$4	-	-	❖	-	❖	❖	❖	❖	❖	❖	❖	❖

❖: biologically active IFN detected by an antiviral bioassay

-: no detection of biologically active IFN by an antiviral bioassay

7.3.2.2.2 Oocyst dose: 10^5 *T. gondii* oocysts

Cell free supernatants from short term culture of PBM cells from sheep and cattle stimulated *in vitro* with *Toxoplasma* antigen were collected and analysed for the presence of IFN by ELISA. The amount of IFNγ detected in these samples was calculated using standards ovine and bovine rIFNγ standards (Table 7. 3, Figure 7. 3 and Figure 7. 4). Data for individual animals are displayed on Table 7. 8, Table 7. 9 and represented in Figure 7. 8 and Figure 7. 9.

More than 2 U/ml of IFNγ was detected in all the samples analysed from infected sheep (Table 7. 8) which were collected on days 2, 5, 12 and 19 after oral infection. In the samples taken from the cattle group on the same days, IFNγ (>1U/ml) was detected from all the animals studied in the experiment (Table 7. 9). Due to insufficient sample volume, it was not possible to determine whether the IFNγ was biologically active.

It is important to mention that similar amounts of IFNγ were detected from all the sheep samples analysed after infection with 10^5 *T. gondii* oocysts, but within the cattle group infected with the same dose, there is variation between individual animals regarding the amount of IFNγ detected.

Supernatants samples from both ovine and bovine cells cultured in medium alone were consistently negative for IFNγ.

Table 7. 8 IFNγ detected in cell free supernatants from short term culture of PBMC from sheep after *in vitro* stimulation with *T. gondii* antigen

Oocyst dose: 10^5 *T. gondii* oocysts					
Animal	**day of infection**	**%OD**	**sd**	**sem**	**U/ml**
$S_3$1	2	0.073	0.01	0.00	2.45
	5	0.065	0.00	0.00	2.45
	12	0.073	0.00	0.00	2.45
	19	0.089	0.00	0.00	2.51
$S_3$2	2	0.070	0.01	0.01	2.45
	5	0.072	0.00	0.00	2.45
	12	0.078	0.00	0.00	2.51
	19	0.105	0.00	0.00	2.63
$S_3$3	2	0.082	0.00	0.00	2.51
	5	0.066	0.00	0.00	2.45
	12	0.069	0.00	0.00	2.45
	19	0.151	0.12	0.08	2.81
$S_3$4	2	0.063	0.00	0.00	2.45
	5	0.074	0.00	0.00	2.45
	12	0.074	0.00	0.00	2.45
	19	0.065	0.00	0.00	2.45
$S_3$5	2	0.075	0.00	0.00	2.45
	5	0.069	0.00	0.00	2.45
	12	0.074	0.00	0.00	2.45
	19	0.150	0.11	0.08	2.81
$S_3$6	2	0.085	0.01	0.00	2.51
	5	0.092	0.01	0.01	2.57
	12	0.072	0.00	0.00	2.45
	19	0.084	0.00	0.00	2.51

Table 7. 9 IFNγ detected in cell free supernatants from short term culture of PBMC from cattle after *in vitro* stimulation with *T. gondii* antigen

Oocyst dose: 10^5 *T. gondii* oocysts						
Animal	**day of infection**	**%OD**	**sd**	**sem**	**pg/ml**	**U/ml**
$C_4$1	2	0.794	0.09	0.06	218.60	4.37
	5	0.609	0.02	0.01	167.53	3.35
	12	0.473	0.02	0.02	130.08	2.60
	19	0.245	0.01	0.01	67.45	1.35
$C_4$2	2	0.070	0.00	0.00	19.13	0.38
	5	0.192	0.01	0.01	52.86	1.06
	12	0.082	0.00	0.00	22.44	0.45
	19	0.261	0.00	0.00	71.86	1.44
$C_4$3	2	0.105	0.01	0.00	28.91	0.58
	5	0.213	0.01	0.00	58.50	1.17
	12	0.251	0.02	0.01	68.97	1.38
	19	0.444	0.02	0.01	122.10	2.44
$C_4$4	2	0.247	0.01	0.01	68.00	1.36
	5	2.311	0.11	0.08	636.10	12.72
	12	0.568	0.09	0.06	156.38	3.13
	19	0.176	0.01	0.00	48.32	0.97
$C_4$5	2	0.135	0.01	0.01	37.03	0.74
	5	0.150	0.00	0.00	41.30	0.83
	12	0.383	0.01	0.00	105.44	2.11
	19	0.090	0.00	0.00	24.78	0.50
$C_4$6	2	0.083	0.00	0.00	22.71	0.45
	5	0.235	0.01	0.01	64.56	1.29
	12	0.465	0.03	0.02	128.02	2.56
	19	0.349	0.02	0.02	95.95	1.92

Figure 7. 8 IFNγ in cell free supernatants from short term culture of ovine PBMC after *in vitro* stimulation with *T. gondii* antigen

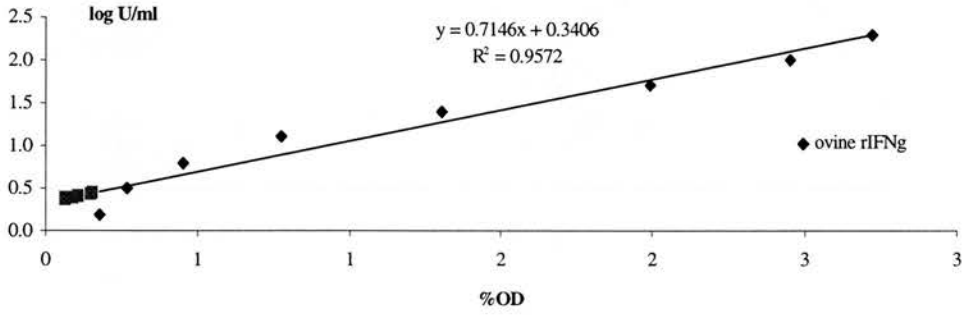
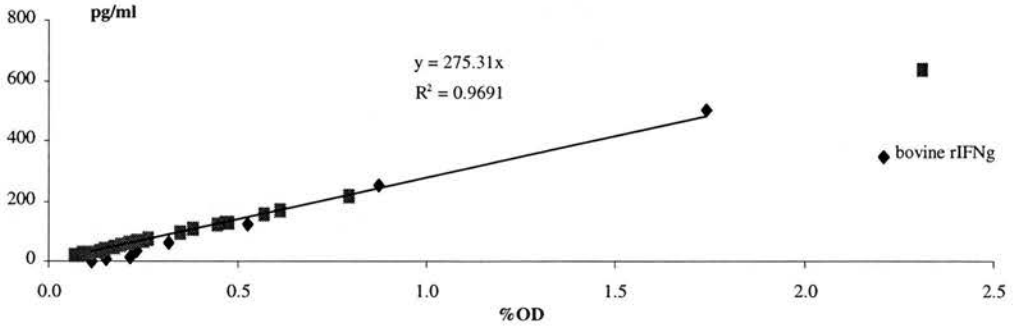


Figure 7. 9 IFNγ detected in cell free supernatants from short term culture of bovine PBMC after *in vitro* stimulation with *T. gondii* antigen



7.3.3 Antigen-induced proliferation assay on peripheral blood mononuclear cells

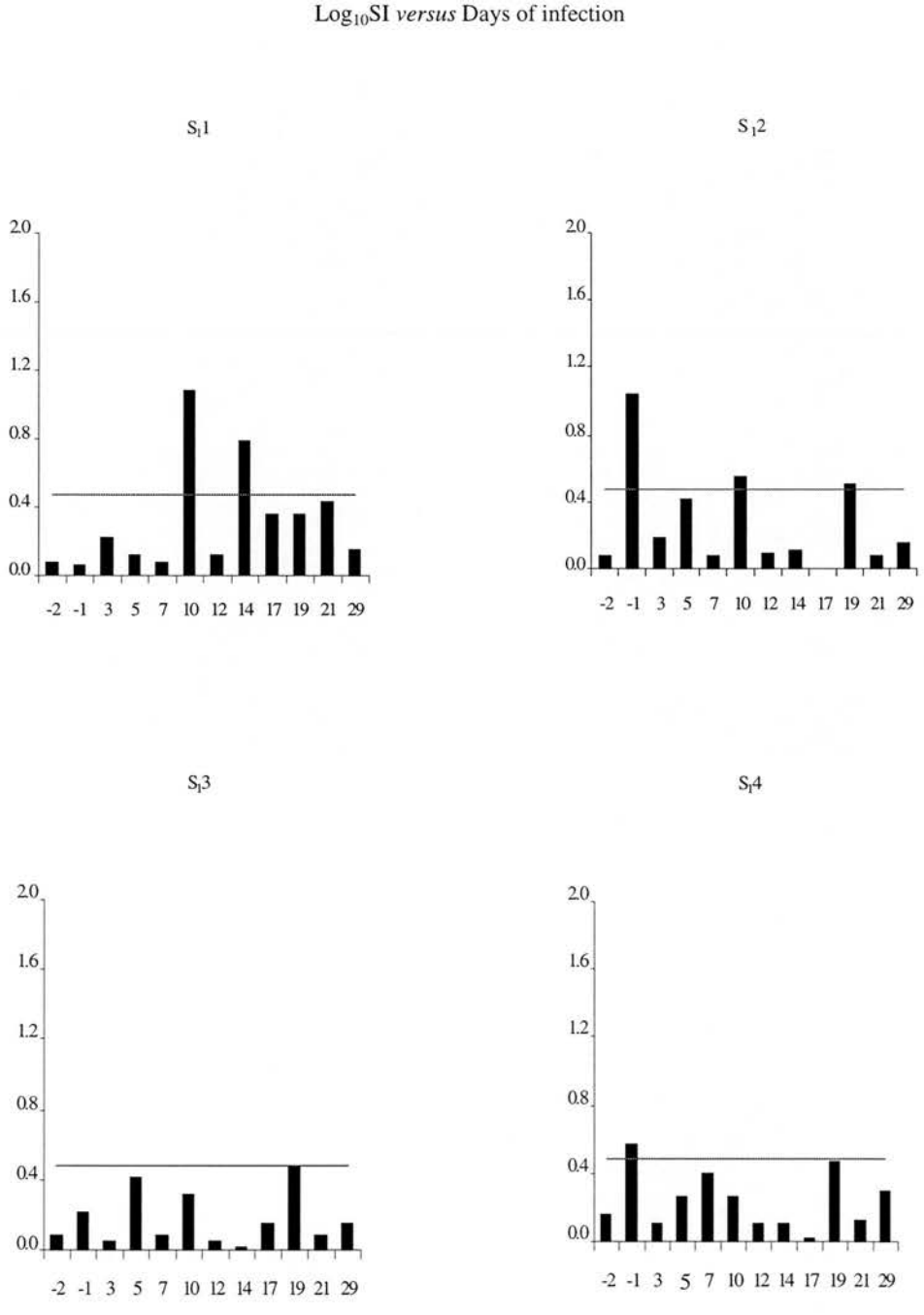
Peripheral blood mononuclear cells were tested for their ability to respond to *T. gondii* antigen to determine at what time after infection antigen-specific cells could be detected in peripheral blood.

The results are expressed as Log_{10} stimulation indices and are illustrated in Figure 7.10 to Figure 7.13. SI were determined by dividing the arithmetic mean of counts per minute (cpm) from the *Toxoplasma* antigen stimulated cultures, pulsed with ^{3} [H]-thymidine, by the arithmetic mean of cpm of the background medium control cultures. The data correspond to individual animals undergoing primary oral infection with either 10^3 or 10^5 oocysts of *T. gondii*. The results are expressed as $Log_{10}SI$ to enable comparison between animals. Actual cpm values for proliferative responses to medium control, Con A control and *T. gondii* antigen are presented in Appendix Chapter 7 Table 7 to Table 26.

In this experiment the test is considered positive when the SI has a value above 2.5-3 according to Kristensen and coworkers (1982). This is represented in the graphs by a continuous line at $Log_{10}3= 0.47$.

Figure 7. 10 illustrates the results obtained from sheep infected with 10^3 oocysts. Variable responses were recorded in the sheep. Two of the animals showed positive proliferation to *T. gondii* antigen on one occasion prior to infection. Two out of the four sheep showed positive proliferation activity to *T. gondii* antigen at 10, 14 and 19 days post infection.

Figure 7. 10 Proliferation of PBMC from sheep following stimulation with 12.5 μg/ml of *T. gondii* antigen.

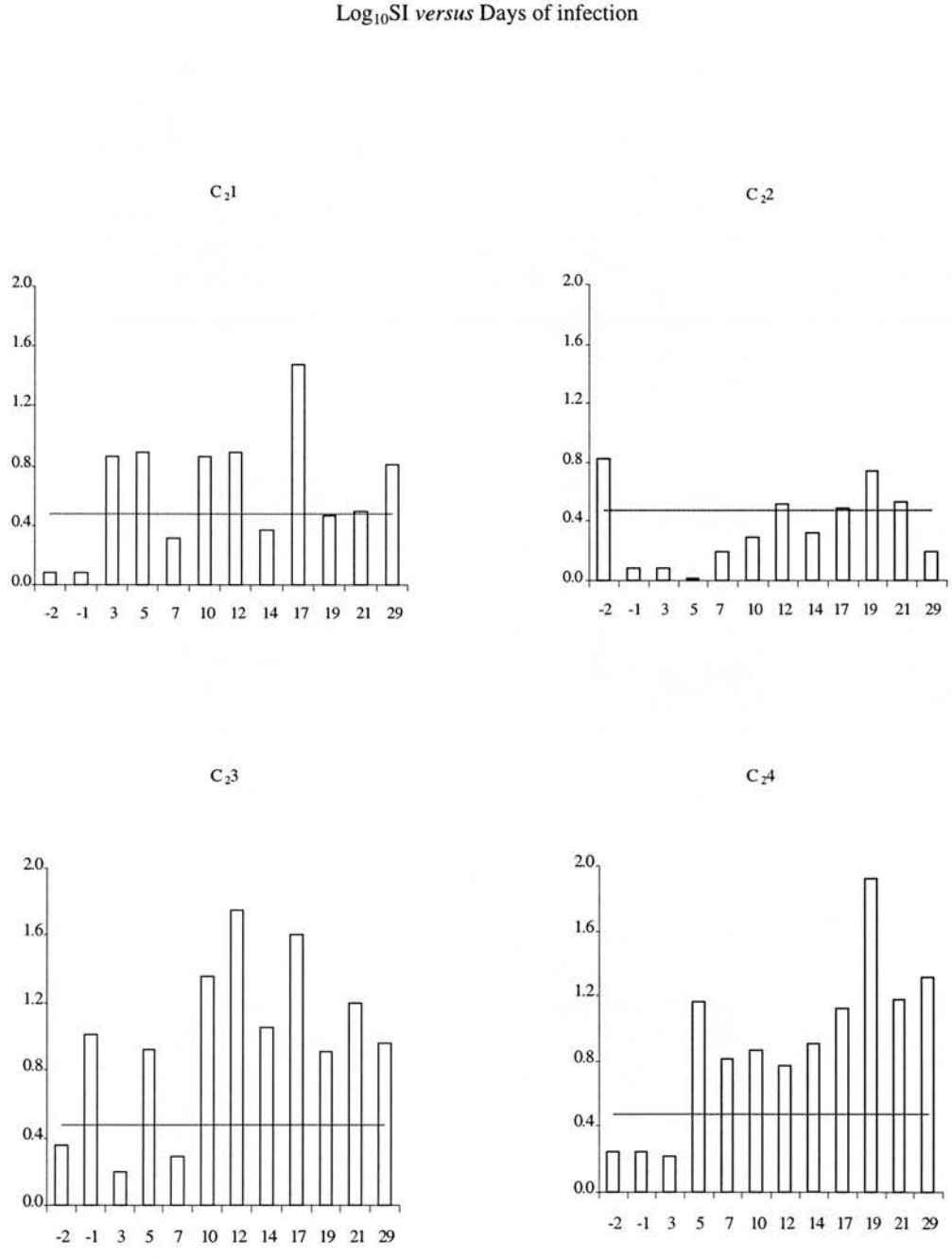


Infective dose: 10^3 *T. gondii* oocysts of M3 isolate. Cut off point: $Log_{10}3 = 0.47$.

Figure 7. 11 illustrates the results obtained from the four cattle infected with 10^3 oocysts. In this case, proliferative responses were more frequently detected with higher SI values compared to the group of sheep receiving a similar dose of oocysts.

Two of the animals ($C_2$2 and $C_2$3) presented SI values above 3 on one occasion prior to infection. All the cattle showed positive SI values (greater than 3) following infection with oocysts, although there was considerable variation in the magnitude of the response between the four animals. The highest SI responses were detected between days 12 and 19 post infection.

Figure 7. 11 Proliferation of PBMC from cattle following stimulation with 12.5μg/ml of *T. gondii* antigen.



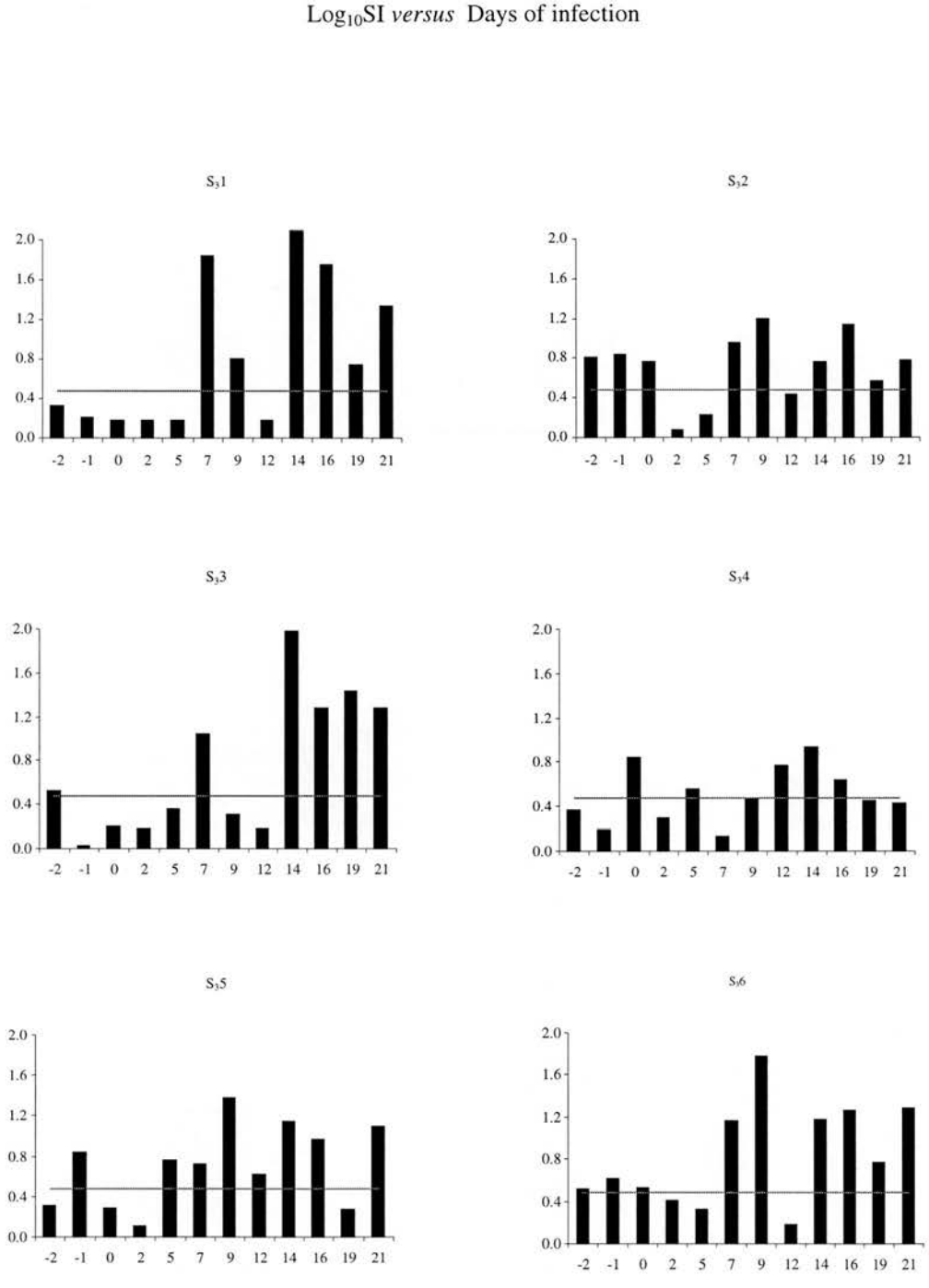
Infective dose: 10^3 *T. gondii* oocysts of M3 isolate. Cut off point: $Log_{10}3 = 0.47$.

Figure 7. 12 and Figure 7. 13 show the proliferation of PBM cells from sheep and cattle orally infected with a high dose of infective oocysts (10^5) of *T. gondii*.

In this case, when the infective dose was increased, greater proliferative responses were recorded and more frequently from the sheep group when compared with the same species receiving the lower infective dose (Figure 7. 12 and Figure 7. 13). Positive proliferation of cells to *T. gondii* antigen was observed in 5 out of 6 sheep by day seven after infection. In the sixth sheep, proliferation to *T. gondii* antigen occurred on day 12-14 after infection. However, occasionally positive proliferative responses to the specific antigen occurred in some of the animals prior to infection.

Figure 7. 13 illustrates the proliferative responses of PBM cells to *T. gondii* antigen in cattle given the high dose of *T. gondii* oocysts (10^5). There was a lot of variation between responses of individual animals. In the majority of cases proliferative responses to *T. gondii* antigen occurred around day 7-9 after infection but in common with the group of sheep there were occasional responses prior to infection. Maximum SI values were registered between days 12 and 14 of the monitored period.

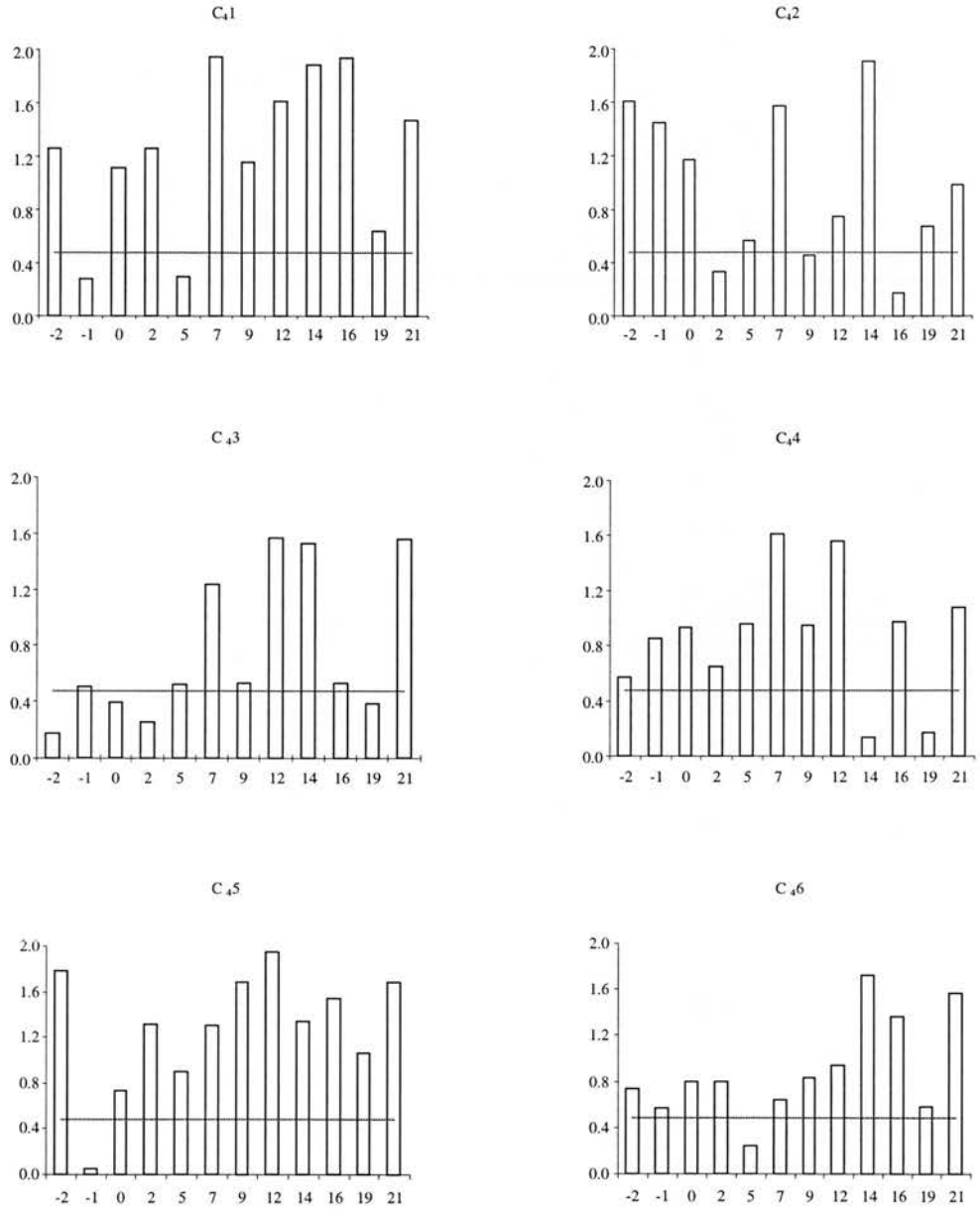
Figure 7. 12 Proliferation of PBMC from sheep to stimulation with 12.5µg/ml of *T. gondii* antigen.



Infective dose: 10^5 *T. gondii* oocysts of M3 isolate. Cut off point: $Log_{10}3 = 0.47$.

Figure 7. 13 Proliferation of PBMC from cattle to stimulation with 12.5 μg/ml of *T. gondii* antigen.

$Log_{10}SI$ *versus* Days of infection



Infective dose: 10^5 *T. gondii* oocysts of M3 isolate. Cut off point: $Log_{10}3= 0.47$.

7.4 Discussion

It is well known that sheep and cattle differ markedly in their vulnerability to peroral *T. gondii* infection acquired for the first time during pregnancy. Studies using experimentally infected mice have shown that cell mediated immunity is important in the host defence to *T. gondii* infection. These findings have been supported by the fact that *Toxoplasma* encephalitis has been observed only in immunocompromised patients with low $CD4^+$ and $CD8^+$ T cell counts and decreased serum levels of IFNγ (Gazzinelli *et al.*, 1993).

In order to increase the understanding of why cattle may be more resistant than sheep to *T. gondii* infection, aspects of the cell mediated immune responses were examined.

Much of the work on host immune responses to *T. gondii* has been carried out on murine models, indicating that both $CD4^+$ and $CD8^+$ T cells are relevant in the development of protective immunity (Suzuki and Remington, 1988; Gazzinelli *et al.*, 1991) and moreover that the $CD8^+$ T cell subset is the major effector population in controlling the infection (Suzuki and Remington, 1988; Parker *et al.*, 1991; Khan *et al.*, 1994). Innes and co-workers,(1995a) demonstrated that the majority of lymphoblast cells in efferent lymph from sheep responding to a primary infection, were T cells. They also reported an increase in the $CD8^+$:$CD4^+$ T cell ratio in lymph during the peak lymphoblast response which coincides with disappearance of the parasite from the efferent lymph. The data presented in this chapter also showed an increase in the percentage of $CD8^+$ cells compared to $CD4^+$cells in the peripheral blood of sheep following oral infection with *T. gondii*. However the timing of appearance of the $CD8^+$ increase reported by Innes and co-workers (1995a) was later than in this study where the percentage of $CD8^+$ T cells increased between days 2 and 5 after infection. This difference may be due to the fact that the

animals used in this study were infected with oocysts, unlike the sheep used by Innes and co-workers (1995a) which received a subcutaneous inoculation of tachyzoites. An increase in the proportion of $CD8^+$ T cells compared to $CD4^+$ T cells in peripheral blood was also reported in human cases of acute toxoplasmosis (Luft *et al.*, 1984; Sklenar *et al.*, 1986). Blackwell and co-workers (1993) showed that mice responding to acute *Toxoplasma* infection had an increase in the number of $CD8^+$ T cells and an increase in the $CD8^+$:$CD4^+$ T cell ratio when they examined spleen cells. They speculated that the increase in the $CD8^+$:$CD4^+$ T cell ratio could be an indication of an immune response in the periphery triggering the parasite to encyst, thus resulting in a larger number of *T. gondii* cysts in the tissues of those animals. Consequently, as there was no obvious increase in the percentage of $CD8^+$ T cells in the cattle it may be interesting to speculate whether this response may be involved in determining tissue cyst development in cattle. However, it is not possible to verify these findings as there are no other studies to my knowledge examining cell mediated immunity to *Toxoplasma* infection in cattle.

The cytokine IFNγ is known to play a decisive role in the outcome of a *T. gondii* infection (Subauste and Remington, 1991). The importance of IFNγ in protection against *T. gondii* infection has been demonstrated in murine infection models. Treatment of mice with recombinant murine IFNγ protected against virulent *Toxoplasma* challenge (McCabe *et al.*, 1984) and depletion of IFNγ in mice by specific monoclonal antibodies abolished resistance to a normally avirulent strain of *T. gondii* (Suzuki *et al.*, 1989). Further studies *in vitro* have shown that IFNγ plays a crucial role in activation of particular cell types, such as macrophages, to inhibit multiplication of the parasite. Thus, treatment of murine peritoneal or alveolar macrophages *in vitro* with recombinant murine IFNγ inhibited intracellular multiplication of *T. gondii* (Black *et al.*, 1987). Human recombinant IFNγ also inhibited *T. gondii* intracellular replication in both macrophages (Nathan *et al.*, 1983) and fibroblasts (Pfefferkon and Guyre, 1984). Similar results were reported by Oura

and collaborators (1993) where ovine recombinant IFNγ inhibited *T. gondii* replication within both ovine fibroblasts and macrophages *in vitro*. However, it was suggested that there was some form of species variation in the capacity of IFNγ to activate particular cell types to inhibit multiplication of the parasite as murine IFNγ failed to inhibit *T. gondii* multiplication within murine fibroblasts *in vitro* (Schwartzman *et al.*, 1990). Gamma interferon is also involved in the maturation of $CD8^+$ cells into effector cytotoxic cells (Zanovello *et al.*, 1988). The data from this study showed that IFN γ was detected more frequently in peripheral blood plasma from the cattle compared to the sheep. Although the titres of IFN detected in the cattle were low. The most probable reason for this is that the samples were collected from peripheral blood and therefore any IFN produced as a result of *T. gondii* infection would be present in very low levels in the periphery. In this study IFN was not detected in sheep peripheral blood plasma. However, a previous study examining the local immune response following *T. gondii* infection in sheep showed high titres of IFNγ in efferent lymph samples from a node draining the site of infection (Innes *et al.*, 1995b).

A recent study conducted at Moredun Research Institute showed that peripheral blood plasma from cattle infected with the closely related parasite *Neospora caninum* did not contain detectable levels of IFNγ but when PBM cells were stimulated with *N. caninum* antigen *in vitro* the cells produced high titre of IFNγ (Innes *et al.*-personal communication). These studies would suggest that measurement of IFN levels directly from blood plasma is not a particularly sensitive or specific method to monitor CMI in large animal species.

When PBM cells from the sheep and cattle were stimulated with *T. gondii* antigen *in vitro*, IFNγ was detected in both groups. It was interesting to observe that in the experiment examining the responses of sheep and cattle given 10^3 *T. gondii* oocysts biologically active IFN was detected more frequently and for a longer period of time in the PBM cells of cattle stimulated with antigen *in vitro* compared to the sheep. Unfortunately there was an

insufficient volume of each sample to do further work in this area. Future studies should aim to collect larger volumes of antigen stimulated cell culture supernatants to allow a more detailed study of the cytokines which may be relevant to the understanding of ovine and bovine immunity to *T. gondii*.

Proliferation of PBM cells to *T. gondii* antigen following *in vitro* stimulation was detected in both cattle and sheep during a primary infection with *T. gondii*. In general a low proliferative response was detected in the group of sheep inoculated with 10^3 oocysts. Whereas the cattle given the same dose of oocysts showed a more pronounced proliferation although there was a large variation between animals within the group. When the dose of oocysts was increased to 10^5 in the second experiment a stronger and more frequent antigen induced cell proliferation was detected in both sheep and cattle. Generally the sheep began to respond on day 7-9 after infection. This timing of detection of this antigen induced cell proliferation is similar to that described by Innes and collaborators (1995a) when examining the proliferation of efferent lymph cells to *T. gondii* antigen *in vitro*, where they demonstrated cell proliferative responses from day 6 following a primary infection. This was also the case when mesenteric lymph node cells from orally infected mice were tested for their ability to respond to *T. gondii* antigen *in vitro* (Chardès *et al.*, 1993).

Cell proliferation in response to *T. gondii* antigen was detected earlier after infection in cattle than in sheep. However, proliferative activity was observed in some of the animals prior to infection. The reasons for this seemingly non-specific proliferative activity are not clear. The cattle and sheep were seronegative for *T. gondii* before infection and therefore it was assumed that these animals were undergoing a primary infection with the parasite. The proliferative activity before infection may indicate some form of cross reactive response to a component of *T. gondii* antigen preparation. Recent experiments examining cell proliferative responses in animals infected with *N. caninum* indicated that there was considerable cross reactivity using crude lysate antigen

preparations of *N. caninum* and *T. gondii* (Khan *et al.*, 1997). This cross reactive response has also been observed in cattle infected with *N. caninum*, where cells proliferated to both *N. caninum* and *T. gondii* lysate antigen, although the cattle were seronegative to *T. gondii* throughout the experiment (Innes *et al.*-personal communication). The cattle in this experiment were seronegative to *N. caninum* before infection but it is not known whether the animals were seronegative for *Sarcocystis spp.* or whether there are cross reactive antigens between *Sarcocystis spp.*, *T. gondii* and *N. caninum*. This may explain some of the seemingly non-specific proliferative activity observed particularly in the cattle, before infection. As very little is known about cell mediated immunity to *Sarcocystis spp.* or the antigens recognized by infected hosts this may be an important area of investigation (Fayer and Dubey, 1984; Gasbarre *et al.*, 1984; Dubey *et al.*, 1989).

Seemingly, non-specific proliferative responses of human lymphocytes from seronegative patients to water soluble *T. gondii* antigen have also been reported (Hughes *et al.*, 1984). This non-specific proliferation was significantly reduced when they used secretory antigens as opposed to water soluble antigens, indicating that the specificity of the antigen used in the proliferation assay is critical in determining the specificity of the proliferative response (Hughes *et al.*, 1984). Also it is known that resting lymphocytes, as a homogeneous population, have a low uniform RNA- and DNA-content with a low incorporation of ^{3}H-thymidine. However, freshly collected lymphocytes may have a very high thymidine uptake, depending on their cell cycle at the time of collection. This activity is sometimes maintained in the *in vitro* culture resulting in high background proliferation (Kristensen *et al.*, 1982).

A phenotypic analysis of the proliferating cells before and after infection may also help to determine the nature of this non-specific proliferation. This would also have been helpful to determine which cells were responsible for the IFNγ production. Stimulation of immune spleen cells *in vitro* with a crude tachyzoite antigen induced IFNγ production, predominantly

from the $CD4^+$ T cell subset (Gazzinelli et al., 1991). Phenotypic analysis of short term cultures of ovine cells also showed that $CD4^+$ T cells were responsible for much of the IFNγ production (Innes and Wastling, 1995).

It has been suggested that $CD4^+$ T cells are an important component of the immune response during the early stages of infection (Araujo, 1991). While $CD8^+$ cells are reported to be the major effector T lymphocyte, their induction and optimal activity are dependent upon $CD4^+$ helper function (Denkers *et al.*, 1993) presumably through the production of Th1 cytokines such as IL-2 and IFN γ. The cattle in this study showed an early increase in $CD4^+$ T cells and showed earlier production of IFN compared to the sheep. It is likely that the outcome of a *T. gondii* infection is determined at a very early stage in the encounter between parasite and host. The early induction of IFNγ seems to be critical to host survival and also will direct the adaptive immune response towards a Th1/$CD8^+$ T cell pathway. Recently, very interesting work has shown that *T. gondii* has the ability to directly activate macrophages to produce cytokines such as IL-12 which will stimulate NK cells to produce IFNγ. Therefore prior to the induction of the adaptive immune response the innate mechanisms are already inhibiting parasite multiplication and providing the correct cytokine environment for the induction of an effective immune response (Gazzinelli *et al.*, 1993).

If cattle are more efficient than sheep as regards the early induction of IFNγ this may help to explain their superior resistance. Comparing the ability of *T. gondii* antigens to directly stimulate cattle and sheep macrophages and examining the resultant cytokines produced may help us to understand this early and perhaps critical immune response.

7.5 Conclusions

Most of the work examining host immune responses to *Toxoplasma* infection has emphasised the strong and persistent CMI response that the parasite elicits, which protects the host against rapid parasite growth and consequent pathology.

In this comparative study cells from both cattle and sheep showed proliferative activity to a crude lysate of *T. gondii in vitro*, although in general the cells from cattle showed a stronger response and were detected more frequently than cells from sheep. IFNγ was also detected more frequently in the cattle samples compared to the sheep.

In summary the cattle seem to develop a more pronounced cell mediated immune response than the sheep following oral infection with either 10^3 or 10^5 *T. gondii* oocysts.

Chapter 8

General discussion and concluding remarks

8. General discussion and concluding remarks

The main aims of the present study were to compare the immune responses and pathogenesis of sheep and cattle following a primary oral infection with *Toxoplasma gondii*. The rationale behind the study was to further our understanding of why these two animal species are different in their vulnerability to *Toxoplasma* infection.

A summary of the main findings of this study is described below:

When sheep and cattle are infected with the same dose of *T. gondii*, the sheep show a more severe temperature response which begins earlier and last longer than that observed in cattle. Parasitaemia is more frequently detected in sheep than in cattle using similar sampling procedures. Also *T. gondii* was detected in the tissues of sheep whereas it was not detected in cattle tissues at post mortem.

These data would suggest that the cattle were better able to control the resultant infection following oral inoculation of *T. gondii* oocysts compared to the sheep.

To further our understanding of the mechanisms which may be important in resistant to *T. gondii*, several aspects of the host immune response were measured in sheep and cattle.

There was a greater increase in specific antibody titre in sheep compared to cattle following infection. The magnitude of the antibody response appeared to correlate with the magnitude of the systemic *T. gondii* infection. In sheep given a titrated dose of oocysts the group given the highest dose (10^5) showed the greatest increase in antibody titre. Therefore as the sheep showed a greater magnitude of specific antibody response than the cattle given the same dose of oocysts, one could speculate that this may reflect the fact that the cattle were better able to control the parasitaemia than the sheep.

When examining cell mediated immunity there was a consistent, early increase in the percentage of $CD4^+$ T cells (from base line values) in the group of cattle following *T. gondii* infection. Also the cytokine IFNγ was more frequently detected in samples of cell free plasma or from cultured cell supernatants in the cattle compared to the sheep. $CD4^+$ T cells have been shown in previous studies to be a major source of the cytokine IFNγ (Gazzinelli *et al.*, 1991). In general, proliferation of cells to a crude lysate antigen of *T. gondii* was more frequently detected and of a higher magnitude in the cattle compared to the sheep.

Therefore the results from the assays conducted in this experiment would suggest that the cell mediated immune responses of cattle were more pronounced than those of the sheep.

8.1 Presence of T. gondii *within tissues*

The study described in this thesis showed that the presence of parasites in tissues was much more frequently detected in sheep compared to cattle following infection of both groups of animals with an equal dose of oocysts. *T. gondii* was more frequently detected in brain and cardiac muscle. The reason why it was easier to detect *T. gondii* in sheep tissues compared to cattle is not known. The sheep and cattle were initially given the same oocyst dose (10^3 or 10^5), however, host immune mechanisms are likely to have a profound effect on parasite multiplication *in vivo*. Therefore, one can assume that if cattle are more efficient at controlling *T. gondii* multiplication early in the infection due to an effective immune response, then less parasite would disseminate to the tissues, and consequently *T. gondii* is less frequently detected in cattle than in sheep. However, it is important to consider the size of the sample examined and to note that a negative result from any sample does not necessarily mean that the whole tissue is free of the presence of the parasite. The problem of sample error when working with large animal species can only be avoided by examining the whole animal, which is clearly impractical. Therefore when

attempting *T. gondii* detection from large animal species, the size of the sample examined along with the specificity and sensitivity of the technique used are important factors to consider and lack of detection may be due to a combination of these two factors and not necessarily to the absence of parasite in the tissues.

8.2 Comparison of techniques to detect T. gondii

The detection of *T. gondii* in tissue sections by histology may prove difficult due to the morphological similarities between *T. gondii* and other closely related parasites such as *N. caninum* and *Sarcocystis* spp. and the low density of parasites within the tissues (Uggla and Buxton, 1990; Cole *et al.*, 1993). Thus, a more sensitive and specific test is required to reveal the presence of *T. gondii* in large animals.

Isolation of *T. gondii* is usually carried out by inoculation of samples into laboratory animals. Cats constitute the ideal model for isolation of *Toxoplasma* due to both high specificity and the possibility of analysing a large sample size (Dubey and Thulliez, 1993). However the drawback of this method is the low availability of seronegative cats required for the isolation of *T. gondii*. Mice are the most commonly used animal when attempting *T. gondii* isolation as they are very sensitive to infection. However, although the isolation of parasites by inoculation of samples into mice is conclusive and specific (Dubey and Frenkel, 1973; Lindberg and Frenkel, 1977; Derouin *et al.*, 1987), it is not possible to inoculate large tissue samples into mice.

The PCR test has been used to detect *T. gondii* DNA from several different samples taken from various animal species (Turner and Savva, 1991; Wastling *et al.*, 1993; Buxton *et al.*, 1994; Steuber *et al.*, 1995; Stiles *et al.*, 1996). Considering the high conservation across strains of *T. gondii* of the B1 multicopy gene (Burg *et al.*, 1989) and the very close agreement between the PCR technique and mouse

inoculation for detection of parasites (Wastling *et al.*, 1993), PCR is a useful alternative for demonstrating the presence of parasite. However, although the PCR is highly sensitive, it does not distinguish whether the parasite material is dead or alive. Inoculation into mice reveals the presence of viable *T. gondii* in the tissues.

8.3 Temperature response

A common feature of *T. gondii* infection is a brief fever response. This has been reported in sheep and cattle following *T. gondii* infection (Buxton *et al.*, 1994; McColgan *et al.,* 1988; Buxton *et al.*, 1991; Costa *et al.*, 1977; Munday, 1978; Dubey, 1983). This study showed that the temperature response occurs earlier in sheep and lasted longer compared to the temperature response in cattle. It is known that the cytokine IL-1 is an endogenous pyrogen and that the prostaglandin E_2 (PGE_2) is involved in suppression of a variety of relevant immune factors (lymphocyte proliferation, cytotoxic T lymphocytes, NK cells and cell to cell interaction). Moreover, induction of both of these immune components is associated with the quantity of antigen *in vivo* which comes in contact with macrophages (Austyn and Wood, 1993). If one assumes that the temperature response induced in animals following infection with *T. gondii* is dependent on IL-1 with the involvement of prostaglandin E_2, then if there are a larger number of parasites and hence antigenic material in the sheep, this may directly provoke the macrophages with associated induction of IL-1 and PGE_2. If there is less antigen in the cattle then presumably there will be less IL-1 and PGE_2 activity which is reflected in a less severe temperature response.

In summary, if cattle are more efficient in controlling early *T. gondii* multiplication compared to sheep, less *Toxoplasma* antigen would circulate in the host, and subsequently a less severe temperature response would be recorded, which fits with the observed findings in this study.

8.4 Humoral immune response

Both sheep and cattle showed specific *T. gondii* antibody responses following infection. The sheep showed a higher increase in specific antibody titre compared to the cattle. It is known that in general terms antibody production is driven by the quantity of antigen *in vivo* and that as the antigen decreases so production of antibody decreases by a simple feedback mechanism (Roitt, 1991). The data from this comparative study reveals further supporting evidence that the cattle were better able to control *T. gondii* establishment and multiplication than the sheep. This is reflected in the relative magnitude of the antibody responses.

The anti-*T. gondii* function of this antibody response was not addressed in this thesis but reports in the literature have suggested various roles for antibody in immunity to *T. gondii*. Specific antibodies can destroy tachyzoites in the presence of complement as was demonstrated when tachyzoites incubated with immune serum revealed distortion of the *Toxoplasma* cytoplasm resulting in poor staining compared with the deep blue stained cytoplasm of *Toxoplasma* incubated with nonimmune serum. This is the basis of the Sabin and Feldman dye test for diagnosis of *T. gondii* which is still held in many test centers as the "gold standard" (Sabin and Feldman, 1948). Tachyzoites opsonized by antibodies or complement are not able to evade the normal killing mechanisms following uptake by macrophages (Joiner *et al.*, 1990). This was demonstrated using viable antibody-coated *T. gondii* tachyzoites incubated with Chinese hamster ovary cells (CHO) transfected with macrophage-lymphocyte Fc receptors. The uptake of the antibody-coated tachyzoites led to the formation of vacuoles capable of both fusion and acidification. It has also been suggested that *Toxoplasma* specific antibodies may inhibit the invasion of cells by blocking the activity of secretory-excretory elements responsible of promoting host cell penetration, like rhoptries or micronemes (Schwartzman, 1986). However, in a primary *T. gondii* infection the kinetics of induction of specific antibody response is relatively slow, specific antibodies first being detected on day 7-8 after infection (Innes and Wastling, 1995). Therefore while antibody may play an important role in

secondary infection, in primary infection its role may take second place to cell mediated or innate immune mechanisms which are induced at an earlier stage in infection.

8.5 Cell mediated immune response

Since *T. gondii* is an obligate intracellular parasite, cell mediated immune mechanisms are thought to be important in resolving primary infection. From the extensive work carried out on the immunity elicited by *T. gondii* in mouse models, T cells and IFNγ are known to be key factors in determining the outcome of infection (Gazzinelli *et al.*, 1991; Subauste and Remington, 1991). The importance of T cells in protective immunity has been revealed through adoptive transfer experiments using murine models (Suzuki and Remington, 1988; Parker *et al.*, 1991). This is corroborated by the recrudescence of latent *T. gondii* infections in patients with AIDS where $CD4^+$ and $CD8^+$ T cell counts fall below certain levels (Luft *et al.*, 1984; Navia *et al.*, 1986).

In this study, cattle showed an early and brief increase in the percentage of $CD4^+$ T cells in peripheral blood and showed stronger and more frequent proliferative activity in PBM cells cultured with specific antigen *in vitro* and IFNγ was more frequently detected in either blood plasma or in activated PBM cells when compared to sheep under the same conditions. Phenotypic analysis of PBMC in sheep undergoing a primary infection showed an increase in the $CD8^+$: $CD4^+$ T cell ratio, which is in agreement with a study reported by Innes *et al.,* (1995a) following experimental inoculation of sheep. They described a similar phenomenon in ovine efferent lymph cells responding to a primary *T. gondii* infection. The cattle showed an early and brief increase in the percentage of $CD4^+$ T cells which was not seen in the sheep. $CD4^+$ T lymphocytes have been shown to have a major role in the development of resistance to the parasite (Suzuki and Remington, 1988; Araujo, 1991). Araujo demonstrated the participation of $CD4^+$ T cells in the development of

resistance to *T. gondii* and in the mechanisms controlling the formation of *T. gondii* cysts in the brains of mice. This activity was revealed when the number of *T. gondii* cysts in the brains of mice treated with a monoclonal antibody against $CD4^+$ was significantly higher than in mice with intact $CD4^+$ populations (Araujo, 1991). Moreover, although it has been suggested that $CD8^+$ T cells are the major effector cells in *T. gondii* infection, their induction and optimal activity is dependent upon $CD4^+$ helper function (Denkers *et al.,* 1993; Innes *et al.*, 1995a).

IFNγ is known to be important in protective immunity to *T. gondii* and has been suggested as the major mediator of host resistance (Suzuki *et al.,* 1988). This cytokine is known to be able to activate several cell types, in particular macrophages to kill multiplying parasites within the cells (McCabe *et al.,* 1984; Murray *et al.,* 1985; Black *et al.,* 1987). IFNγ has also been reported to promote the maturation of $CD8^+$ T cells into effector cytotoxic cells (Zanovello *et al.,* 1988). $CD4^+$ and $CD8^+$ T cells are known to produce IFNγ in response to stimulation with *T. gondii* (Gazzinelli *et al.,* 1993), although *in vitro* at least, $CD4^+$ T cells seem to be the major producers of IFNγ. Interestingly, recent studies have demonstrated that NK cells can produce IFNγ very early in *T. gondii* infection prior to the induction of specific cell mediated immune responses (Hauser *et al.*, 1982, 1983; Sher *et al.,* 1993). Previous studies in sheep examining cell free efferent lymph plasma have shown the presence of IFNγ in animals undergoing primary and secondary infection with *T. gondii* (Innes *et al.,* 1995b). The presence of IFNγ has been also observed in supernatants of primed mesenteric lymph node cells in mice following an oral *T. gondii* infection (Chardès *et al.,* 1993). However, both of these studies were examining local immune responses and not responses in the periphery. This study showed that in general IFN was more frequently detected in cattle than in sheep, however the quantity of IFN detected was comparatively low compared to that reported by Innes and collaborators (1995b). A possible explanation for this may be that cytokines are likely to be found at a comparatively higher concentration in local immune responses than in the periphery. Therefore a study of the cytokine response following *T. gondii* infection is probably best achieved by examining a local immune response and in the case of orally

infected animals it would be best to examine responses in the gut. However, measuring the kinetics of cytokine responses locally in the gut of large animal species such as cattle and sheep would pose considerable difficulties.

The results from this thesis have largely described and compared the immune responses of cattle and sheep following a primary oral infection with *T. gondii*. Studies on the anti-*T. gondii* mechanisms of various components of the immune response would be the next step to help determine why cattle are better able to control the infection than sheep.

8.6 Concluding remarks and future studies

The results arising from the present study indicate that resistance to oral *Toxoplasma* infection is likely to mediated by a number of immune mechanisms acting together. It is difficult to draw any precise conclusion as to why cattle may be less vulnerable to *T. gondii* infection than sheep. In general the cattle appear to be better able to control the parasitaemia resulting in fewer parasites being detected in their tissues compared with sheep. Interestingly the cytokine IFNγ was more frequently detected either from plasma or activated cell supernatants in the infected cattle compared to the sheep. This cytokine is known to play an important role in protection against *T. gondii* and has been implicated as a factor involved in the differentiation of tachyzoites to bradyzoites (tissue cyst stage). Therefore if IFNγ is a critical factor in determining the outcome of a *T. gondii* infection, future studies should focus on the mechanisms of induction of IFNγ within species of differing vulnerability to *T. gondii*.

In oral *Toxoplasma* infection, the first line of host defence is in the intestinal mucosa surface, which consists of epithelial cells, intraepithelial lymphocytes (mainly $CD8^+$ phenotype), mucus and secretory immunoglobulins (IgA) (Chardès *et al.*, 1993). Increasing evidence suggests that intraepithelial lymphocytes play a major

role at the mucosal surfaces as a first line of defence against enteric invasion (Chardès *et al.,* 1994) The mucosal effector mechanism has been characterized as cytotoxic T cell activity directed against *T. gondii*-infected-epithelial cells, which are then eliminated from the epithelium. Defined mucosal immunity can be elicited with the presence of *T. gondii* immunogens, which are potent inducers of local immune response via IgA production, T cell stimulation and cytokine secretion. Studies on the early mucosal immune response elicited following oral *Toxoplasma* infecting in cattle might elucidate the cause of the efficiency of cattle in controlling parasite multiplication.

Several studies have clearly demonstrated the unusual capacity of *T. gondii* to nonspecifically activate macrophages as well as natural killer cells leading to the early production of cytokines as IFNγ and TNF-α during initial stages of infection before specific T cell induction (Hauser *et al.,* 1982, 1983; Gazzinelli *et al.,* 1993). It is believed that an early stimulation of both of these cell populations by the parasite is crucial in establishing an early nonspecific resistance against the rapidly dividing tachyzoites before development of an adaptive T cell mediated immune response (Gazzinelli *et al.,* 1993). The early production of IFNγ by NK cells has also been implicated in the biasing of the $CD4^+$ T cell response towards the Th1 phenotype, which produces the inflammatory cytokines, IL-2 and IFNγ known to be important in protective immunity to *T. gondii*.

In conclusion, the information arising from the present study and from the relevant literature on *T. gondii* infection, suggest that cattle are better able to control *T. gondii* multiplication, and subsequently reducing the number of parasites disseminated to the tissues probably via a combination of effective immune mechanisms.

The study of the early induction of IFNγ by innate mechanisms may provide the answer as to why cattle are more resistant than sheep or other more vulnerable species to *T. gondii* infection.

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Appendix Chapter 4

Appendix Chapter 4 Table 1 Animals orally infected with a titrated dose of sporulated *T. gondii* oocysts

Group	M3 oocyst dose	Animal number	Species	Breed	Sex	Age
1	10^5	S1	Ovine	Grey face	male	5 months
		S2	"	"	"	"
		S3	"	"	"	"
		S4	"	"	"	"
2	10^4	S5	"	"	"	"
		S6	"	"	"	"
		S7	"	"	"	"
		S8	"	"	"	"
3	10^3	S9	"	"	"	10 months
		S10	"	"	"	5 months
		S11	"	"	"	"
		S12	"	"	"	"

Appendix Chapter 4 Table 2 Rectal temperatures before and after oral infection of sheep given 10^5 *T. gondii* oocysts of the M3 isolate.

days of infection	S1	S2	S3	S4	mean	sd*	sem*
-2	39.78	39.55	40.10	40.23	39.92	0.31	0.15
-1	39.75	39.88	40.19	39.94	39.94	0.18	0.07
0	39.68	39.63	40.13	40.25	39.92	0.31	0.13
1	40.05	40.14	40.01	40.25	40.11	0.11	0.05
2	38.89	40.18	40.05	40.60	39.93	0.73	0.15
3	41.83	41.69	39.80	40.49	40.95	0.98	0.40
4	42.51	42.48	42.09	42.26	42.32	0.20	0.08
5	42.25	42.45	42.53	42.61	42.46	0.15	0.04
6	41.65	42.05	42.2	42.24	42.04	0.27	0.05
7	41.15	41.53	41.19	41.60	41.37	0.23	0.09
8	41.31	41.20	41.35	41.97	41.46	0.35	0.17
9	39.99	41.69	40.03	40.78	40.62	0.80	0.34
10	39.61	40.36	39.52	40.07	39.89	0.40	0.18
11	39.59	41.46	39.76	40.04	40.21	0.85	0.38
12	39.49	39.85	39.65	40.16	39.79	0.29	0.11
13	39.85	39.78	39.84	39.93	39.85	0.06	0.03
14	39.66	40.15	39.88	39.89	39.90	0.20	0.07

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 4 Table 3 Rectal temperatures after oral infection of sheep given 10^4 *T. gondii* oocysts of the M3 isolate

days of infection	S5	S6	S7	S8	mean	sd*	sem*
-2	39.48	40.37	38.94	39.58	39.59	0.59	0.29
-1	39.70	39.71	39.58	39.73	39.68	0.07	0.03
0	39.77	39.65	39.51	39.33	39.57	0.19	0.07
1	39.39	39.72	39.43	39.85	39.60	0.22	0.09
2	39.93	39.85	39.83	39.83	39.86	0.05	0.01
3	39.85	39.81	39.53	39.69	39.72	0.14	0.06
4	41.66	41.68	40.03	41.94	41.33	0.87	0.42
5	42.40	42.20	42.51	42.47	42.40	0.14	0.07
6	41.97	42.21	42.41	42.17	42.19	0.18	0.06
7	41.69	41.84	41.71	41.68	41.73	0.07	0.03
8	40.66	41.33	41.57	40.37	40.98	0.56	0.26
9	40.94	41.21	41.63	41.20	41.25	0.29	0.10
10	39.94	40.55	40.37	39.95	40.20	0.31	0.13
11	39.38	39.60	40.12	39.57	39.67	0.32	0.13
12	39.63	39.17	39.71	39.68	39.55	0.25	0.12
13	39.83	39.53	39.77	39.57	39.68	0.15	0.05
14	39.42	39.57	39.65	39.445	39.52	0.11	0.04

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 4 Table 4 Rectal temperatures after oral infection of sheep given 10^3 *T. gondii* oocysts of the M3 isolate

days of infection	S9	S10	S11	S12	mean	sd*	sem*
-2	39.65	39.46	39.93	39.83	39.72	0.21	0.10
-1	39.12	40.13	39.92	39.26	36.61	0.49	0.19
0	39.69	39.77	39.72	39.91	39.77	0.10	0.04
1	39.87	39.87	39.89	40.35	40.00	0.24	0.11
2	39.59	39.88	40.06	40.09	39.91	0.23	0.05
3	39.66	39.58	39.56	39.34	39.54	0.14	0.06
4	39.49	39.95	40.25	39.81	39.88	0.32	0.10
5	41.44	41.38	41.42	42.04	41.57	0.31	0.15
6	42.12	42.29	41.94	42.17	42.13	0.15	0.07
7	41.94	41.99	42.08	41.69	41.93	0.17	0.08
8	41.01	41.25	41.44	41.33	41.26	0.18	0.04
9	41.03	41.75	41.37	40.99	41.29	0.35	0.16
10	39.95	42.25	40.52	40.73	40.86	0.98	0.39
11	39.48	41.96	39.02	39.21	39.92	1.37	0.67
12	39.81	39.11	39.37	39.85	39.54	0.36	0.15
13	39.51	39.30	39.64	39.77	39.56	0.20	0.10
14	39.32	39.47	39.41	39.45	39.41	0.07	0.01

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 4 Table 5 Reciprocal IFAT titres of IgG antibody in sheep infected with 10^5 *T. gondii* oocysts of the M3 isolate

days of infection	S1	S2	S3	S4	mean	sd*	sem*
-2	0	32	16	32	16	2.38	1.19
0	0	0	16	16	4	2.31	1.15
2	0	0	0	0	0	0	0
4	32	32	32	32	32	0	0
6	32	32	16	32	32	0.50	0.25
8	64	32	1/32	32	32	0.50	0.25
10	128	64	64	64	64	0.50	0.25
12	1024	1024	1024	1024	1024	0	0
14	1024	1024	1024	1024	1024	0	0

Appendix Chapter 4 Table 6 Reciprocal IFAT titres of IgG antibody in sheep infected with 10^4 *T. gondii* oocysts of M3 isolate

days of infection	S5	S6	S7	S8	mean	sd*	sem*
-2	16	16	16	16	16	0	0
0	16	16	16	16	16	0	0
2	32	32	32	16	32	0.50	0.25
4	32	32	32	32	32	0	0
6	32	64	32	32	32	0.50	0.25
8	32	64	32	32	32	0.50	0.25
10	64	64	32	64	64	0.50	0.25
12	1024	1024	1024	1024	1024	0	0
14	256	1024	1024	1024	1024	1	0.5

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 4 Table 7 Reciprocal IFAT titres of IgG antibody in sheep infected with 10^3 *T. gondii* oocysts of M3 isolate

days of infection	S9	S10	S11	S12	mean	sd*	sem*
-2	16	16	16	16	16	0	0
0	16	16	16	16	16	0	0
2	32	16	32	32	32	0.50	0.25
4	32	16	32	32	32	0.50	0.25
6	32	16	32	32	32	0.50	0.25
8	16	64	256	64	64	1.63	0.82
10	16	16	32	32	32	0.58	0.29
12	256	1024	256	512	512	0.96	0.48
14	512	1024	256	512	512	0.82	0.41

Appendix Chapter 4 Table 8 % OD detected by IgG-ELISA in serum from sheep infected with 10^5 *T. gondii* oocysts of M3 isolate

days of infection	S1	S2	S3	S4	mean	sd*	sem*
-2	10	10	10	10	10	0	0
0	10	10	10	10	10	0	0
2	10	10	10	10	10	0	0
4	10	10	10	10	10	0	0
6	10	10	10	10	10	0	0
8	10	10	10	10	10	0	0
10	21	10	10	10	12.75	5.50	0.69
12	33	18	16	30	24.25	8.50	3.18
14	38	38	21	37	33.50	8.35	3.91
19	50	89	52	81	68	19.92	8.09

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 4 Table 9 % OD detected by IgG-ELISA in serum from sheep infected with 10^4 *T. gondii* oocysts of M3 isolate

days of infection	S5	S6	S7	S8	mean	sd*	sem*
-2	10	16	10	16	13	3.46	1.44
0	10	10	10	19	12.25	4.50	2.13
2	10	15	10	18	13.25	3.95	1.63
4	10	17	10	17	13.50	4.04	1.68
6	10	17	10	15	13.00	3.56	1.49
8	10	10	10	15	11.25	2.50	1.18
10	10	19	10	21	15	5.83	2.43
12	20	23	21	40	26	9.42	4.29
14	30	33	38	48	37.25	7.89	3.18
19	44	52	76	86	64.5	19.76	7.34

Appendix Chapter 4 Table 10 % OD detected by IgG-ELISA in serum from sheep infected with 10^3 *T. gondii* oocysts of M3 isolate

days of infection	S9	S10	S11	S12	mean	sd*	sem*
-2	10	10	17	10	11.75	3.50	1.66
0	10	10	19	10	12.25	4.50	2.13
2	10	10	20	10	12.50	5	2.37
4	10	10	18	10	12	4	1.89
6	10	10	17	10	11.75	3.50	1.66
8	10	10	18	10	12	4	1.89
10	10	10	10	19	12.25	4.50	2.13
12	10	10	28	19	16.75	8.62	3.72
14	10	21	42	32	26.25	13.82	4.50
19	36	78	52	61	56.75	17.54	5.66

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 6

Appendix Chapter 6 Table 1 Rectal temperatures before and after primary oral infection of sheep given 10^3 *T. gondii* oocysts of the M3 isolate

days of infection	$S_1$1	$S_1$2	$S_1$3	$S_1$4	mean	sd*	se*
-3	38.7	39.85	39.53	39.9	39.50	0.55	0.28
-2	39.87	39.54	40.25	40.27	39.98	0.35	0.17
-1	40.13	39.6	39.69	40.31	39.93	0.34	0.17
0	40.01	39.63	39.62	40.09	39.84	0.25	0.12
1	39.59	39.85	40.05	39.88	39.84	0.19	0.09
2	39.77	39.27	39.62	39.55	39.55	0.21	0.10
3	39.78	39.46	39.94	39.61	39.70	0.21	0.10
4	40.83	40.54	40.85	41.1	40.83	0.23	0.11
5	41.53	41.5	41.65	41.45	41.53	0.08	0.04
6	41.6	41.65	41.44	41.68	41.59	0.11	0.05
7	41.05	41.01	41.45	41.37	41.22	0.22	0.11
8	40.89	40.78	41.33	40.88	40.97	0.25	0.12
9	39.96	39.44	40.77	40.01	40.05	0.55	0.27
10	39.65	39.25	39.45	39.25	39.40	0.19	0.10
11	39.44	39.17	39.45	39.3	39.34	0.13	0.07
12	39.72	39.44	39.59	39.67	39.61	0.12	0.06
13	39.21	39.2	39.49	39.26	39.29	0.14	0.07
14	39.48	39.09	39.57	39.49	39.41	0.22	0.11

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 6 Table 2 Rectal temperatures before and after primary oral infection of cattle given 10^3 *T. gondii* oocysts of the M3 isolate

days of infection	C_21	C_22	C_23	C_24	mean	sd*	se*
-3	38.81	38.8	38.27	38.37	38.56	0.28	0.14
-2	38.68	38.91	38.69	39.04	38.83	0.18	0.09
-1	38.78	38.99	38.75	38.92	38.86	0.11	0.06
0	38.61	40.54	39.03	38.98	39.29	0.85	0.43
1	39.07	39.28	38.64	38.85	38.96	0.28	0.14
2	38.93	38.86	39.06	38.21	38.77	0.38	0.19
3	38.07	39.01	38.65	38.69	38.61	0.39	0.20
4	39.01	38.77	38.77	39.02	38.89	0.14	0.07
5	38.81	40.37	39.77	40.33	39.82	0.73	0.36
6	40.93	40.25	40.88	40.15	40.55	0.41	0.20
7	40.92	39.77	40.77	40.01	40.37	0.56	0.28
8	40.25	39.09	39.99	39.77	39.78	0.50	0.25
9	39.23	38.57	39.16	38.98	38.99	0.30	0.15
10	38.61	38.72	38.77	39.06	38.79	0.19	0.10
11	38.47	38.69	38.69	38.55	38.60	0.11	0.05
12	38.44	38.85	38.79	38.89	38.74	0.21	0.10
13	38.67	38.8	38.61	38.65	38.68	0.08	0.04
14	38.74	38.61	38.68	38.93	38.74	0.14	0.07

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 6 Table 3 Rectal temperatures before and after primary oral infection of sheep given 10^5 *T. gondii* oocysts of the M3 isolate

days of infection	$S_3$1	$S_3$2	$S_3$3	$S_3$4	$S_3$5	$S_3$6	mean	sd*	sem*
-3	39.93	40.25	39.61	38.97	39.93	39.36	39.68	0.46	0.19
-2	39.45	39.57	39.63	39.6	39.49	39.34	39.51	0.11	0.05
-1	39.71	39.4	39.69	39.71	39.65	39.55	39.62	0.12	0.05
0	40.32	39.36	39.41	39.01	39.62	39.33	39.51	0.44	0.18
1	39.46	39.27	39.25	38.98	39.45	38.79	39.20	0.27	0.11
2	40.02	39.49	39.73	38.96	39.97	39.95	39.69	0.41	0.17
3	40.91	41.05	40.94	39.32	41.04	41.1	40.73	0.69	0.29
4	41.45	41.64	41.93	41.2	41.57	41.78	41.60	0.26	0.11
5	41.7	41.12	41.55	41.37	41.59	41.19	41.42	0.23	0.10
6	40.89	41.04	41.33	41.47	41.49	41.15	41.23	0.24	0.10
7	40.98	41.08	41.05	40.93	41.37	40.45	40.98	0.30	0.13
8	40.89	40.13	41.34	39.63	41.12	40.05	40.53	0.68	0.28
9	39.41	38.52	40.68	38.97	40.34	38.53	39.41	0.92	0.38
10	39.56	39.17	39.69	38.84	38.68	39.49	39.24	0.41	0.17
11	39.49	39.01	39.18	39.21	39.09	39.19	39.20	0.16	0.07
12	38.85	39.08	39.09	38.57	38.97	39.2	38.96	0.23	0.09
13	39.37	39.17	39.17	39.21	39.25	39.16	39.22	0.08	0.03
14	39.51	39.13	39.36	39.13	39.32	39.11	39.26	0.16	0.07

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 6 Table 4 Rectal temperatures before and after primary oral infection of cattle given 10^5 *T. gondii* oocysts of the M3 isolate

days of infection	$C_4$1	$C_4$2	$C_4$3	$C_4$4	$C_4$5	$C_4$6	mean	sd	sem
-3	38.08	37.63	37.66	38.26	38.2	38.48	38.05	0.34	0.14
-2	38.53	38.89	38.65	38.73	38.69	38.34	38.64	0.19	0.08
-1	37.99	38.26	39.01	38.56	38.79	38.38	38.50	0.37	0.15
0	38.33	38.21	38.18	38.85	38.47	38.17	38.37	0.26	0.11
1	38.81	38.34	38.42	38.65	38.44	38.53	38.53	0.17	0.07
2	38.44	38.64	38.7	38.77	38.73	38.23	38.59	0.21	0.09
3	39.47	40.61	39.01	40.13	38.78	38.89	39.48	0.74	0.31
4	39.91	40.3	40.36	39.49	40.84	39.28	40.03	0.58	0.24
5	40.32	40.89	40.87	38.37	39.8	40.72	40.16	0.97	0.40
6	39.69	40.29	40.09	38.48	39.81	40.29	39.78	0.68	0.28
7	38.65	39.54	39.19	37.99	39	39.9	39.05	0.67	0.28
8	38.48	39.22	38.13	37.92	37.94	38.65	38.39	0.50	0.21
9	37.18	38.23	38.2	37.94	37.09	37.76	37.73	0.50	0.21
10	37.65	38.23	37.82	37.89	37.61	37.87	37.85	0.22	0.09
11	37.42	38.39	38.53	38.38	38.5	38.53	38.29	0.43	0.18
12	38.97	38.26	37.41	38.43	38.13	38.28	38.25	0.50	0.21
13	37.5	38.3	38.37	37.9	38.17	38.31	38.09	0.34	0.14
14	38.11	38.24	38.4	38.22	37.47	38.18	38.10	0.32	0.14

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 6 Table 5 Reciprocal IFAT titres of IgG antibody in sheep infected with 10^3 *T. gondii* oocysts of M3 isolate

days of infection	$S_1$1	$S_1$2	$S_1$3	$S_1$4	mean	sd*	se*
-3	16	16	16	16	16	0.00	0.00
-2	16	16	16	16	16	0.00	0.00
-1	16	16	16	16	16	0.00	0.00
0	16	16	16	16	16	0.00	0.00
3	16	16	16	5	16	0.50	0.25
5	32	32	16	64	32	0.82	0.41
7	16	16	16	64	32	1.00	0.50
10	128	16	64	256	64	1.71	0.85
12	1024	1024	1024	2048	1024	0.50	0.25
14	8192	2048	1024	2048	2048	1.26	0.63
17	8192	2048	1024	8192	4096	1.50	0.75
19	16384	4096	1024	16384	8192	1.91	0.96
21	8192	4096	2048	16384	8192	1.29	0.65
28	16384	4096	8192	8192	8192	0.82	0.41
182	512	256			512	0.71	0.50
210	512	512			512	0.00	0.00
238	256	256			256	0.00	0.00
266	256	256			256	0.00	0.00
301	256	256			256	0.00	0.00
329	256	256			256	0.00	0.00
371	1024	256			512	1.41	1.00

***sd: standard deviation, sem: standard error of the mean**

Sheep $S_1$3 and $S_1$4 were killed at 6 weeks after infection and postmortem examination was carried out

Appendix Chapter 6 Table 6 Reciprocal IFAT titres of IgG antibody in cattle infected with 10^3 *T. gondii* oocysts of M3 isolate

days of infection	$C_2$1	$C_2$2	$C_2$3	$C_2$4	mean	sd*	se*
-3	256	256	64	128	128	0.48	0.48
-2	256	256	64	128	128	0.48	0.48
-1	256	256	64	128	128	0.96	0.48
0	256	256	64	128	128	0.96	0.48
3	256	256	128	128	256	0.58	0.29
5	256	256	128	128	256	0.58	0.29
7	256	256	128	512	256	0.82	0.41
10	256	256	256	512	256	0.50	0.25
12	1024	256	8256	1024	9512	1.15	0.58
14	512	512	1024	1024	1024	0.58	0.29
17	512	1024	1024	1024	1024	0.50	0.25
19	512	1024	1024	1024	1024	0.50	0.25
21	1024	1024	1024	1024	1024	0.00	0.00
28	1024	1024	1024	1024	1024	0.00	0.00
182		4096		2048	4096	0.71	0.50
210		4096		4096	4096	0.00	0.00
238		4096		4096	4096	0.00	0.00
266		2048		4096	4096	0.71	0.50
301		2048		1024	2048	0.71	0.50
329		2048		8192	4096	1.41	1.00
371		2048		2048	2048	0.00	0.00

***sd: standard deviation, sem: standard error of the mean**

Cattle $C_2$1 and $C_2$3 were killed at 6 weeks after infection and postmortem examination was carried out

Appendix Chapter 6 Table 7 Reciprocal IFAT titres of IgG antibody in sheep infected with 10^5 *T. gondii* oocysts of M3 isolate

Days of infection	$S_3$1	$S_3$2	$S_3$3	$S_3$4	$S_3$5	$S_3$6	mean	sem*	sd*
-3	32	64	128	64	128	128	64	0.33	0.82
-2	32	64	128	64	128	128	64	0.33	0.82
-1	32	64	128	64	128	128	64	0.33	0.82
0	32	128	128	64	256	128	128	0.42	1.03
3	32	128	128	128	256	128	128	0.40	0.98
5	32	128	128	128	512	128	128	0.52	1.26
7	64	128	128	128	512	256	128	0.42	1.03
10	512	256	512	512	2048	1024	512	0.42	1.03
12	1024	512	1024	1024	2048	4096	1024	0.42	1.03
14	2048	2048	2048	8192	8192	4096	4096	0.40	0.98
21	4096	8192	2048	8192	8192	8192	8192	0.34	0.84
28	8192	4096	16384	8192	16384	8192	8192	0.31	0.75
36	8192	4096	16384	16384	16384	16384	16384	0.34	0.84
43	8192	8192	16384	16384	32768	16384	16384	0.31	0.75
73				16384	8192	16384	16384	0.33	0.58
103				8192	4096	4096	4096	0.33	0.58
133				8192	2048	4096	4096	0.58	1.00
166				8192	8192	4096	8192	0.33	0.58

***sd: standard deviation, sem: standard error of the mean**

Sheep $S_3$1, $S_3$2 and $S_3$4 were killed at 6 weeks after infection and postmortem examination was carried out

Appendix Chapter 6 Table 8 Reciprocal IFAT titres of IgG antibody in cattle infected with 10^5 *T. gondii* oocysts of M3 isolate

Days of infection	C_41	C_42	C_23	C_44	C_45	C_46	mean	sd*	sem*
-3	128	128	128	128	128	128	128	0.00	0.00
-2	128	128	128	128	128	128	128	0.00	0.00
-1	128	128	128	128	128	128	128	0.00	0.00
0	128	128	128	128	128	128	128	0.00	0.00
3	128	256	128	128	128	128	128	0.41	0.17
5	128	256	256	128	128	128	128	0.52	0.21
7	128	256	256	128	128	512	256	0.82	0.33
10	1024	256	8192	256	256	512	512	1.97	0.80
12	1024	1024	16384	256	256	1024	1024	2.19	0.89
14	1024	2048	16384	256	512	1024	1024	2.07	0.84
21	2048	4096	16384	512	512	2048	2048	1.90	0.77
28	8192	16384	16384	8192	8192	16384	16384	0.55	0.22
36	16384	16384	16384	8192	8192	16384	16384	0.52	0.21
43	8192	16384	32768	8192	8192	16384	16384	0.82	0.33
73				4096	8192	16384	8192	1.00	0.58
103				1024	2048	8192	2048	1.53	0.88
133				1024	2048	8192	2048	1.53	0.88
166				4096	2048	4096	4096	0.58	0.33

***sd: standard deviation, sem: standard error of the mean**

Cattle C_41, C_42 and C_43 were killed at 6 weeks after infection and postmortem examination was carried out

Appendix Chapter 7

Tables 1 to 6 display the percentage of PBMC staining with monoclonal antibodies recognizing $CD2^+$, $CD4^+$ and $CD8^+$ T cell subsets following a primary oral infection of sheep and cattle with 10^5 *T. gondii* sporulated oocysts, where **sd** is standard deviation, **sem** is standard error of the mean and **nd**: not done because of lack of sample

Appendix Chapter 7 Table 1 Percentage of PBMC staining with monoclonal antibodies recognizing $CD2^+$ subsets following a primary oral infection of sheep with 10^5 *T. gondii* oocysts

	$CD2^+$								
Days of infection	**S_31**	**S_32**	**S_33**	**S_34**	**S_35**	**S_36**	**mean**	**sd***	**sem***
-3	0.36	15.93	13.34	8.62	12.85	15.34	11.07	5.85	2.39
-1	18.08	14.3	26.33	12.63	20.61	33.53	20.91	7.86	3.21
0	50.25	9.5	13.92	26.32	15.93	67.29	30.53	23.19	9.47
2	-16.34	7.96	20.77	24.83	34.28	14.87	19.84	9.08	3.71
5	46.27	33.56	19.61	52.52	53.25	54.36	43.26	13.94	5.69
7	65.5	90.83	54.87	63.04	62.65	70.21	67.85	12.31	5.03
9	48.88	45.12	61.92	29.92	50.4	65.05	50.22	12.62	5.15
12	26.37	34.58	64.56	28.22	44.35	42.93	40.17	14.03	5.73
14	75.89	61.11	76.56	78.17	n d	64.3	71.21	7.89	3.53
16	41.19	13.79	50.54	12.23	19.42	28.13	27.55	15.52	6.34
19	9.78	10.17	nd	5.83	9.08	11.74	9.32	2.18	0.98
21	33.49	18.33	30.64	39.93	38.78	44.3	34.24	9.17	3.75

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 7 Table 2 Percentage of PBMC staining with monoclonal antibodies recognizing $CD4^+$ subsets following a primary oral infection of sheep with 10^5 *T. gondii* oocysts

	$CD4^+$								
Days of infection	**S_31**	**S_32**	**S_33**	**S_34**	**S_35**	**S_36**	**mean**	**sd***	**sem***
-3	9.97	8.95	8.38	3.15	7.67	10.47	8.10	2.63	1.07
-1	17.63	14.21	25.63	12.15	25.49	35.1	21.70	8.64	3.53
0	5.86	8.78	13.59	24.74	9.97	21.96	14.15	7.60	3.10
2	10.99	8.34	20.77	16.68	25.57	0.19	13.76	9.15	3.73
5	22.53	13.31	24.4	26.16	24.33	29.66	23.40	5.50	2.25
7	37.39	23.56	44.78	22.37	44.66	35.88	34.77	9.85	4.02
9	49.18	48.76	57.87	32.74	50.18	65.79	50.75	11.03	4.50
12	24.31	37.78	64.2	33.88	48.19	40.51	41.48	13.63	5.56
14	77.09	40.33	69.75	64.5	nd	59.45	62.22	13.87	6.20
16	41.41	14.75	47.06	12.22	24.48	26.87	27.80	14.01	5.72
19	11.59	11.58	nd	9.14	12.95	14.69	11.99	2.04	0.91
21	27.1	16.57	31.22	22.04	30.05	36.03	27.17	6.96	2.84

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 7 Table 3 Percentage of PBMC staining with monoclonal antibodies recognizing $CD8^+$ subset following a primary oral infection of sheep with 10^5 *T. gondii* oocysts

	$CD8^+$								
Days of infection	**S_31**	**S_32**	**S_33**	**S_34**	**S_35**	**S_36**	**mean**	**sd***	**sem***
-3	8.25	4.69	5.63	2.33	52	5.83	13.12	19.14	7.81
-1	6.19	52.72	7.99	3.81	12.95	8.9	15.43	18.52	7.56
0	45.81	4.11	6.59	15.78	13.67	52.1	23.01	20.65	8.43
2	2.24	10.66	18.71	5.29	9.94	2.06	8.15	6.34	2.59
5	48.55	6.21	12.73	56.02	42	51.24	36.13	21.24	8.67
7	65.32	62.12	48.2	71.29	54.83	70.38	62.02	9.05	3.69
9	16.28	57.59	56.75	44.11	44.23	44.49	43.91	14.94	6.10
12	19.37	9.97	19.7	14.36	15.06	15.21	15.61	3.60	1.47
14	68.93	56.37	44.42	70.13	nd	43.51	56.67	12.79	5.72
16	28.59	8.01	23.63	11.12	14.24	14.19	16.63	7.85	3.21
19	12.93	5.09	nd	6.98	4.7	4.1	6.76	3.61	1.62
21	27.22	17.66	24.6	55.84	42.6	39.24	34.53	13.99	5.71

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 7 Table 4 Percentage of PBMC staining with monoclonal antibodies recognizing $CD2^+$ subset following a primary oral infection of cattle with 10^5 *T. gondii* oocysts

	$CD2^+$								
Days of infection	**$C_4$1**	**$C_4$2**	**$C_4$3**	**$C_4$4**	**$C_4$5**	**$C_4$6**	**mean**	**sd***	**sem***
-3	67.57	76.15	78.21	71.63	66.21	58.02	69.63	7.36	3.00
-1	61.4	53.68	62.27	59.71	nd	44.95	56.402	7.23	3.23
0	70.9	65.8	75.01	65.42	85.57	71.29	72.33	7.43	3.03
2	26.32	20.75	24.07	23.18	16.83	21.35	22.08	3.26	1.33
5	41.74	69.01	77.08	79.68	94.39	93.21	75.85	19.35	7.90
7	41.07	38.38	91.17	61.83	54.43	51.97	56.47	19.09	7.79
9	77.73	62.33	72.04	77.17	66.37	61.1	69.45	7.28	2.97
12	66.06	70.96	70.15	72.68	68.9	62.72	68.57	3.63	1.48
14	70.66	44.17	71.3	54	75.7	63.8	63.27	12.04	4.91
16	55.07	33.4	43.13	59.86	48.56	45.18	47.53	9.32	3.81
19	33.64	22.13	31.63	49.83	30.13	26.81	32.36	9.46	3.86
21	81.79	57.56	62.03	63.05	54.79	58	62.87	9.76	3.98

***sd: standard deviation, sem: standard error of the mean**

Appendix Chapter 7 Table 5 Percentage of PBMC staining with monoclonal antibodies recognizing $CD4^+$ subset following a primary oral infection of cattle with 10^5 *T. gondii* oocysts

	$CD4^+$								
Days of infection	**C_41**	**C_42**	**C_43**	**C_44**	**C_45**	**C_46**	**mean**	**sd***	**sem***
-3	67.57	35.16	29.18	17.06	20.33	22.67	31.99	18.60	7.59
-1	61.4	32.18	29.57	28.06	nd	21.13	34.46	15.60	6.98
0	70.9	45.44	37.26	32.96	56.12	48	48.44	13.69	5.59
2	26.32	18.56	13.71	11.11	13.21	11.26	15.69	5.86	2.39
5	48.72	50.26	62.22	63.07	89.17	74.37	64.63	15.26	6.23
7	41.07	75.82	86.83	42.19	58.1	56.14	60.02	18.24	7.45
9	77.73	38.32	30.1	33.7	33.53	25.69	39.84	19.03	7.77
12	66.06	34.81	27.55	24.38	26.79	23.5	33.84	16.28	6.65
14	70.66	39.2	34.65	27.55	34.64	30.3	39.5	15.78	6.44
16	55.07	17.74	18.51	25.62	22.97	22.52	27.07	14.03	5.73
19	33.64	8.21	9.16	7.87	11.3	10.7	13.48	9.97	4.07
21	81.79	44.1	43.2	35.51	41.91	37.68	47.36	17.19	7.02

Appendix Chapter 7 Table 6 Percentage of PBMC staining with monoclonal antibodies recognizing $CD8^+$ subset following a primary oral infection of cattle with 10^5 *T. gondii* oocysts

	$CD8^+$								
Days of infection	**$C_4$1**	**C_4**	**$C_4$3**	**$C_4$4**	**$C_4$5**	**$C_4$6**	**mean**	**sd***	**sem***
-3	10.59	17.58	8.41	27.12	3.06	10.61	12.89	8.39	3.42
-1	40.24	12.73	20.64	20.07	nd	13.07	21.35	11.20	5.01
0	24.7	23.4	30.32	25.04	40.57	41.56	30.93	8.20	3.35
2	5.17	12.33	16.43	18.41	18.04	10.52	13.48	5.16	2.11
5	15.25	12.34	17.06	6.23	14.1	10.85	12.63	3.82	1.56
7	9.79	8.43	16.89	20.1	19.56	18.34	15.51	5.10	2.08
9	29.1	16.92	26.33	28.15	16.8	24.79	23.68	5.49	2.24
12	17.47	8.33	16.75	19.14	16.7	12.87	15.21	3.95	1.61
14	16.01	15.71	20.7	22.2	17.06	20.24	18.65	2.74	1.12
16	16.1	15.71	16.55	21.03	17.89	22.47	18.29	2.81	1.15
19	7.55	7.15	7.62	24.74	12.2	9.16	11.40	6.79	2.77
21	30.29	20.31	22.21	28.15	29.15	26.27	26.06	3.99	1.63

***sd: standard deviation, sem: standard error of the mean**

Tables 7 to 26 display the proliferative response of peripheral blood mononuclear cells during primary oral infection of sheep and cattle with either 10^3 or 10^5 *T. gondii* sporulated oocysts.

Mean count per minute (cpm) (n=4) responses of PBMC to *in vitro* stimulation with Con A (positive control) or *T. gondii* antigen from sheep ($S_1$1, $S_1$2, $S_1$3, $S_1$4, $S_3$1, $S_3$2, $S_3$3, $S_3$4, $S_3$5 and $S_3$6) and cattle ($C_2$1, $C_2$2, $C_2$3, $C_2$4, $C_4$1, $C_4$2, $C_4$3, $C_4$4, $C_4$5 and $C_4$6) on days before and after primary oral infection with either 10^3 or 10^5 oocyst of a complete strain (M3) of *T. gondii.*

SI= Stimulation index

Medium= Medium as negative control

Appendix Chapter 7 Table 7 Proliferative response of PBMC during primary oral infection of sheep with 10^3 *T. gondii* oocysts

$S_1$1

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpm x 10^3 ± sem	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value
-2	0.66 ± 0.05	15.13 ± 9.74	23	0.23	0.33 ± 0.01	0.5	10^{-4}	0.61 ± 0.07	0.92	0.58
-1	0.67 ± 0.05	35.55 ± 1.3	53	10^{-4}	0.76 ± 0.08	1.13	0.36	0.65 ± 0.06	1	0.7
3	0.66 ± 0.16	66.90 ± 2.86	101	2×10^{-4}	1.09 ± 0.01	1.65	0.025	0.94 ± 0.06	1.42	0.02
5	0.82 ± 0.13	11.20 ± 1.06	13.66	23×10^{-4}	1.06 ± 0.42	1.3	0.6	0.99 ± 0.02	1.20	0.004
7	18.88 ± 0.67	1.25 ± 0.08	0.66	0	1.7 ± 0.06	0.1	0	1.7 ± 0.15	0.01	0
10	0.09 ± 0.04	9.04 ± 3.02	104	0.067	1.10 ± 0.02	12.22	0.002	1.10 ± 0.06	12.22	0.048
12	0.84 ± 0.06	17.50 ± 1.47	20.83	15×10^{-4}	1.09 ± 0.07	1.3	0.04	0.94 ± 0.01	1.12	0.009
14	1.62 ± 0.2	41.80 ± 1.67	25.80	2×10^{-4}	9.99 ± 1.16	6.16	0.005	4.08 ± 0.81	2.52	0.06
17	0.22 ± 0.03	12.14 ± 0.44	55.18	10^{-4}	0.05 ± 0.11	2.27	0.08	0.34 ± 0.04	1.54	0.064
19	0.46 ± 0.13	3.37 ± 0.4	7.33	0.005	1.04 ± 0.44	2.26	0.28	0.48 ± 0.1	1.04	0.85
21	0.78 ± 0.09	38.98 ± 2.66	49.97	7×10^{-4}	2.07 ± 0.19	2.65	0.007	1.73 ± 0.33	2.22	0.07
29	0.77 ± 0.06	14.78 ± 0.66	19.19	2×10^{-4}	1.1 ± 0.13	1.43	0.09	0.87 ± 0.06	1.13	0.21

Appendix Chapter 7 Table 8 Proliferative response of sheep PBMC during primary oral infection with 10^3 *T. gondii* oocysts

$S_1$2

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5 μg/ml			6.25μg/ml		
Days of infection	cpm x 10^3 ± sem	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value
-2	1.45 ± 0.62	21.93 ±14.74	15.12	0.26	0.53 ± 0.23	0.36	0.027	0.77 ± 0.31	0.53	0.12
-1	0.51 ± 0.04	38.18 ± 2.5	74.86	6×10^{-4}	5.58 ± 1.5	11	0.043	2.55 ± 0.35	5	0.01
3	0.78 ± 0.23	43.80 ± 3.37	56.15	0.001	1.20 ± 0.58	1.54	0.52	0.8 ± 0.09	1	0.84
5	0.78 ± 0.045	18.80 ± 8.42	24.10	0.28	2 ± 0.52	2.56	0.26	2.52 ± 1.26	3.23	0.4
7	11.38 ± 1.22	19.50 ± 8.80	1.71	0.53	10.52 ± 0.11	0.92	0.08	5.49 ± 4.65	0.48	0.43
10	0.59 ± 0.05	29.12 ± 6.85	49.35	0.025	2.07 ± 0.35	3.5	0.025	1.07 ± 0.23	1.81	0.13
12	0.37 ± 0.05	31.01 ± 1.63	83.81	3×10^{-4}	0.45 ± 0.024	1.22	0.06	0.53 ± 0.038	1.43	0.03
14	0.5 ± 0.02	21.08 ± 4.09	42.16	0.015	0.65 ± 0.08	1.3	0.15	0.57 ± 0.08	1.14	0.39
17	0.21 ± 0.02	11.96 ± 0.16	56.95	0	0.21 ± 0.04	1	0.99	0.23 ± 0.024	1.09	0.35
19	0.38 ± 0.06	6.43 ± 1.52	16.92	0.03	1.23 ± 0.87	3.24	0.41	0.32 ± 0.06	0.84	0.4
21	0.30 ± 0.03	77.87 ± 2.76	259	10^{-4}	0.3 ± 0.03	1	0.9	0.36 ± 0.05	1.2	0.3
29	0.29 ± 0.03	8.92 ± 1.41	30.75	88×10^{-4}	0.4 ± 0.1	1.4	0.33	0.34 ± 0.01	1.17	0.013

Appendix Chapter 7 Table 9 Proliferative response of sheep PBMC during primary oral infection with 10^3 *T. gondii* oocysts

	$S_1$3									
	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5µg/ml			12.5µg/ml			6.25µg/ml		
Days of infection	cpm x 10^3 ± sem	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	P value
-2	3.22 ± 1.27	69.07 ± 3.37	21.45	0.033	3.86 ± 0.69	1.2	0.42	3.83 ± 1.1	1.2	0.62
-1	0.88 ± 0.27	46.94 ± 14.87	53.34	0.053	1.43 ± 0.42	1.62	0.28	0.57 ± 0.09	0.64	0.054
3	0.89 ± 0.05	73.2 ± 1.74	82.24	0	0.99 ± 0.04	1.11	0.09	0.82 ± 03	0.92	0.11
5	0.83 ± 0.06	25.23 ± 3.98	30.39	$87x\ 10^{-4}$	2.14 ± 0.72	2.58	0.17	1 ± 0.07	1.2	0.09
7	1 ± 0.07	21.84 ± 0.55	21.84	0	0.94 ± 0.06	0.94	0.47	1.1 ± 0.1	1.1	0.41
10	0.74 ± 0.06	21.18 ± 44	28.62	$36x\ 10^{-4}$	1.5 ± 0.23	2.03	0.047	1.37 ± 0.2	1.85	0.046
12	0.58 ± 0.04	47.10 ± 0.7	81.20	0	0.64 ± 0.06	1.10	0.41	0.58 ± 0.04	1	0.96
14	1.08 ± 0.11	75.08 ± 1.5	69.52	0	1.12 ± 0.11	1.03	0.71	1.58 ± 0.27	1.46	0.16
17	0.12 ± 0.02	14.76 ± 0.39	123	0	0.17 ± 0.04	1.41	0.27	0.17 ± 0.04	1.41	0.27
19	0.15 ± 0.03	1.10 ± 0.26	7.33	0.038	0.45 ± 0.29	3	0.37	0.14 ± 0.01	0.93	0.38
21	0.42 ± 0.11	55.19 ± 8.05	131	$38x\ 10^{-4}$	0.37 ± 0.01	0.88	0.019	0.64 ± 0.09	1.52	0.11
29	0.75 ± 0.03	27.10 ± 1.33	36.13	$3x\ 10^{-4}$	1.05 ± 0.4	1.4	0.5	0.85 ± 0.2	1.13	0.64

Appendix Chapter 7 Table 10 Proliferative response of sheep PBMC during primary oral infection with 10^3 *T. gondii* oocysts

S_14

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpm x 10^3 ± sem	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value
-2	0.74 ± 0.46	41.47 ± 1.36	56	10^{-4}	1.05 ± 0.25	1.42	0.31	1.10 ± 0.13	1.48	0.071
-1	0.36 ± 0.06	32.60 ± 2.05	90.55	0.04	1.31 ± 0.16	3.64	0.11	0.45 ± 0.04	1.25	0.27
3	0.69 ± 0.08	64.01 ± 4.88	92.77	0.001	0.88 ± 0.05	1.27	0.03	0.65 ± 0.17	0.94	0.81
5	0.83 ± 0.05	28.29 ± 2.83	34.08	0.002	1.54 ± 0.55	1.85	0.29	0.78 ± 0.11	0.94	0.72
7	0.79 ± 0.04	31.58 ± 0.84	39.97	0	1.97 ± 0.2	2.5	86 x10^{-4}	1.73 ± 0.17	2.2	0.013
10	0.54 ± 0.02	49.17 ± 0.58	91.05	0	0.98 ± 0.09	1.81	0.018	1.19 ± 0.45	2.20	0.24
12	0.56 ± 0.04	25.85 ± 3.73	46.16	66 x10^{-4}	0.71 ± 0.04	1.27	0.026	0.49 ± 0.06	0.87	0.32
14	0.68 ± 0.03	7.64 ± 0.85	11.23	38 x10^{-4}	0.86 ± 0.1	1.26	0.19	0.74 ± 0.11	1.09	0.63
17	0.15 ± 0.02	9.39 ± 1.04	62.6	0.003	0.16 ± 0.02	1.06	0.8	0.18 ± 0.02	1.2	0.23
19	0.28 ± 0.04	3.63 ± 0.75	12.96	0.021	0.83 ± 0.32	2.96	0.18	0.35 ± 0.02	1.25	0.033
21	0.39 ± 0.08	48.94 ± 5.16	125.5	0.002	0.52 ± 0.09	1.33	0.24	0.67 ± 0.17	1.72	0.19
29	0.32 ± 0.01	16.93 ± 1.84	53	0.003	0.61 ± 0.08	2	0.04	0.42 ± 0.05	1.31	0.18

Appendix Chapter 7 Table 11 Proliferative response of PBMC during a primary oral infection of cattle with 10^3 *T. gondii* oocysts

$C_2$1

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5µg/ml			12.5µg/ml			6.25µg/ml		
Days of infection	cpm x 10^3 ± sem	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value
-2	9.28 ± 2.90	52.28 ± 1.30	5.63	10^{-4}	11.13 ± 9.81	1.20	0.86	33.52 ± 6.34	3.61	0.031
-1	36.56 ± 1.23	44.32 ± 14.53	1.21	0.068	27.97 ± 7.15	0.76	0.042	26.45 ± 9.15	0.72	0.088
3	1.02 ± 0.35	53.49 ± 1.94	52.43	10^{-4}	7.48 ± 0.56	7.33	14 x 10^{-4}	7.87 ± 1.37	7.71	0.016
5	1.63 ± 0.33	28.67 ± 0.84	17.59	10^{-4}	12.80 ± 2.83	7.85	0.029	19.65 ± 2.60	12.05	61 x 10^{-4}
7	7.01 ± 3.28	39.02 ± 4.90	5.56	72 x 10^{-4}	14.63 ± 1.86	2.08	0.026	10.97 ± 1.06	1.56	0.033
10	7.88 ± 0.91	90.75 ± 4.81	11.51	4 x 10^{-4}	57.40 ± 1.68	7.28	10^{-4}	48.20 ± 2.30	6.12	4 x 10^{-4}
12	3.27 ± 0.43	91.05 ± 4.35	27.84	3 x 10^{-4}	25.62 ± 6.74	7.83	0.045	39.97 ± 3.57	12.22	0.002
14	17.11 ± 4.87	55.86 ± 4.06	3.26	24 x 10^{-4}	40.79 ± 6.94	2.38	0.042	38.43 ± 5.50	2.25	0.03
17	0.17 ± 0.01	19.70 ± 0.29	116	0	5.15 ± 0.45	30.29	16 x 10^{-4}	3.32 ± 1.08	19.53	0.061
19	0.16 ± 0.03	15.91 ± 0.38	99.43	0	0.47 ± 0.22	2.93	0.24	0.43 ± 0.16	2.68	0.18
21	0.42 ± 0.05	37.41 ± 0.70	89.07	0	1.30 ± 0.46	3.09	0.16	3.78 ± 0.66	9	0.015
29	0.76 ± 0.09	59.38 ± 5.28	78.13	16 x 10^{-4}	4.85 ± 1.26	6.38	0.048	9.49 ± 2.37	12.48	0.035

Appendix Chapter 7 Table 12 Proliferative response of cattle PBMC during primary oral infection with 10^3 *T. gondii* oocysts

$C_2$2

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpm x 10^3 ± sem	cpm x 10^3 ±sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value
-2	4.92 ± 12.30	46.02 ± 6.67	9.35	0.66	32.72 ± 14.10	6.65	0.33	35.85 ± 9.45	7.28	0.25
-1	57.67 ± 3.54	29.21 ± 2.62	0.5	17 x 10^{-4}	30.86 ± 2.20	0.53	12 x 10^{-4}	44.32 ± 3.70	0.77	0.036
3	72.90 ± 3.80	35.56 ± 0.74	0.48	0	67.07 ± 6.95	0.92	0.46	70.50 ± 5.67	0.96	0.70
5	35.00 ± 3.48	40.04 ± 3.46	1.14	0.24	36.05 ± 4.40	1.03	0.83	35.90 ± 4.07	1.02	0.84
7	13.13 ± 3.26	16.17 ± 1.04	1.23	0.06	20.80 ± 2.40	1.58	0.05	17.33 ± 2.14	1.32	0.14
10	25.40 ± 6.42	31.66 ± 4.24	1.24	0.24	50.29 ± 5.47	1.98	0.02	40.43 ± 7.82	1.59	0.15
12	10.50 ± 0.74	87.40 ± 2.88	8.32	10^{-4}	35.12 ± 5.32	3.34	8 x 10^{-4}	39.66 ± 2.10	3.77	0.02
14	24.40 ± 4.96	47.16 ± 5.15	1.93	0.02	50.72 ± 5.30	2.07	0.02	23.43 ± 3.91	0.96	0.82
17	4.05 ± 1.36	17.41 ± 0.74	4.30	4 x 10^{-4}	12.68 ± 2.67	3.13	0.08	6.75 ± 1.63	1.66	0.20
19	1.76 ± 0.76	15.81 ± 0.48	8.98	10^{-4}	9.67 ± 1.13	5.49	0.006	7.83 ± 0.80	4.44	0.004
21	2.63 ± 0.54	48.92 ± 5.41	18.60	0.003	8.89 ± 2.52	3.38	0.089	16.16 ± 2.99	6.14	0.02
29	27.24 ± 10.70	67.20 ± 5.77	2.46	0.006	43.14 ± 2.63	1.58	0.009	40.26 ± 7.79	1.47	0.19

Appendix Chapter 7 Table 13 Proliferative response of cattle PBMC during primary oral infection with 10^3 *T. gondii* oocysts

$C_2$3

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5µg/ml			12.5µg/ml			6.25µg/ml		
Days of infection	cpm x 10^3 ± sem	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value	cpm x 10^3 ± sem	SI	*P* value
-2	17.42 ± 6.50	43.67 ± 1.19	2.50	2×10^{-4}	40.19 ± 5.26	2.30	0.023	28.47 ± 9.23	1.63	0.32
-1	2.24 ± 0.73	33.29 ± 8.37	14.86	0.034	23.37 ± 2.88	10.43	52×10^{-4}	20.33 ± 6.51	9.07	0.069
3	1.30 ± 0.93	55.13 ± 3.43	42.40	6×10^{-4}	2.07 ± 0.85	1.59	0.43	2.83 ± 0.56	2.17	0.072
5	0.55 ± 0.06	35.39 ± 4.04	64.34	33×10^{-4}	4.58 ± 0.99	8.32	0.027	4.78 ± 0.81	8.69	0.014
7	1.60 ± 0.79	46.02 ± 0.43	28.76	62×10^{-4}	3.08 ± 0.15	1.93	0.063	6.98 ± 2.98	4.36	0.32
10	2.31 ± 0.68	83.08 ± 4.82	35.96	5×10^{-4}	51.76 ± 1.70	22.40	10^{-4}	39.35 ± 13.26	17.03	0.068
12	0.69 ± 0.11	89.67 ± 1.26	130	0	38.74 ± 2.75	56.14	8×10^{-4}	38.53 ± 2.22	55.84	4×10^{-4}
14	4.59 ± 0.82	43 ± 2.16	9.37	4×10^{-4}	52.21 ± 5.62	11.37	35×10^{-4}	59.80 ± 3.35	13	5×10^{-4}
17	0.44 ± 0.20	14.66 ± 1.41	33.32	21×10^{-4}	17.88 ± 1.11	40.63	6×10^{-4}	14.85 ± 0.84	33.75	4×10^{-4}
19	0.27 ± 0.07	15.73 ± 0.71	58.25	2×10^{-4}	2.17 ± 0.43	8.03	0.02	3.60 ± 0.75	13.33	0.02
21	0.99 ± 0.12	26.57 ± 2.25	26.83	15×10^{-4}	15.41 ± 2.05	15.56	59×10^{-4}	35.14 ± 4.93	35.49	62×10^{-4}
29	0.17 ± 0.02	69.19 ± 3.88	407	4×10^{-4}	1.53 ± 0.30	9	0.02	1.39 ± 0.51	8.17	0.099

Appendix Chapter 7 Table 14 Proliferative response of cattle PBMC during primary oral infection with 10^3 *T. gondii* oocysts

$C_2$4

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5µg/ml			12.5µg/ml			6.25µg/ml		
Days of infection	cpmx10^3 ± sem	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	26.54 ± 3.41	38.49 ± 2.58	1.45	0.019	47.11 ± 3.66	1.77	0.011	41.34 ± 3.88	1.55	0.03
-1	8.93 ± 1.79	23.86 ± 1.59	2.67	26 x 10^{-4}	15.88 ± 2.36	1.77	0.06	17.93 ± 2.71	2	0.045
3	38.07 ± 10.68	57.98 ± 2.45	1.52	0.08	63.55 ± 2.40	1.67	0.06	52.56 ± 0.96	1.38	0.042
5	2.76 ± 0.7	44.96 ± 5.28	16.28	41 x 10^{-4}	39.76 ± 3.52	14.40	18 x 10^{-4}	30.30 ± 5.28	10.97	0.014
7	2.71 ± 0.73	34.20 ± 1.19	12.62	10^{-4}	17.82 ± 3.04	6.57	0.016	18.35 ± 4.71	6.77	0.045
10	5.71 ± 1.29	27.77 ± 1.96	4.86	15 x 10^{-4}	41.81 ± 3.23	7.32	15x 10^{-4}	37.99 ± 1.94	6.65	5 x 10^{-4}
12	9.60 ± 4.82	86.65 ± 1.77	9.02	0	57.13 ± 11.55	5.95	0.025	52.68 ± 6.03	5.48	54 x 10^{-4}
14	13.18 ± 2.73	51.29 ± 3.81	3.89	21 x 10^{-4}	105.7 ± 2.82	8.02	10^{-4}	104 ± 4.33	7.89	2 x 10^{-4}
17	1.36 ± 0.56	19.48 ± 0.48	14.32	0	18.05 ± 0.71	13.27	2x 10^{-4}	15.11 ± 1.41	11.11	23 x 10^{-4}
19	0.13 ± 0.02	17.09 ± 0.46	131.5	0	10.89 ± 0.95	83.76	15x 10^{-4}	6.77 ± 1.38	51.53	0.017
21	2.69 ± 0.48	60.87 ± 5.48	22.63	18 x 10^{-4}	40.82 ± 4.24	15.17	29x 10^{-4}	40.46 ± 8.12	15	0.02
29	1.22 ± 0.27	85.57 ± 5.38	70.14	6 x 10^{-4}	24.88 ± 3.30	20.4	0.005	20.63 ± 2.14	17	28 x 10^{-4}

Appendix Chapter 7 Table 15 Proliferative response of sheep PBMC during primary oral infection with 10^5 *T. gondii* oocysts

$S_3$1

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpmx10^3 ± sem	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	0.22 ± 0.02	5.31 ± 1.1	23.89	0.0091	0.48 ± 0.1	2.15	0.041	0.73 ± 0.2	3.31	0.037
-1	0.07 ± 0.01	7.69 ± 0.42	112.89	0.0002	0.11 ± 0.014	1.61	0.0097	0.19 ± 0.02	1.59	0.019
0	0.3 ± 0.13	7.80 ± 0.27	25.77	0	0.28 ± 0.031	0.91	0.0072	0.26 ± 0.05	0.88	0.028
2	0.18 ± 0.01	3.46 ± 0.052	18.80	0	0.135 ± 0.023	0.73	0.61	0.12 ± 0.015	0.69	0.81
5	0.07 ± 0.03	6.86 ± 0.75	88.46	0.0014	0.074 ± 0.005	0.96	0.025	0.077 ± 0.013	1.00	0.12
7	0.07 ± 0.01	7.09 ± 0.2	97.36	0	5.05 ± 0.54	69.41	0.0013	4.92 ± 0.19	67.69	0.0001
9	0.16 ± 0.1	12.48 ± 0.8	76.90	0.0002	1.02± 0.19	6.29	0.0068	0.265 ± 0.07	1.63	0.025
12	0.03 ± 0.002	8.11 ± 0.22	263.21	0	0.012 ± 0.002	0.38	0.99	0.094 ± 0.029	3.06	0.044
14	0.08 ± 0.01	13.96 ± 1.52	172.73	0.0014	9.79 ± 0.97	121.13	0.0011	6.97 ± 0.9	86.23	0.0022
16	0.05 ± 0.006	8.22 ± 0.44	166.08	0.0002	2.79± 0.47	56.49	0.0049	3.45 ± 1.4	69.78	0.046
19	0.36 ± 0.03	3.26 ± 0.26	9.14	0.0008	1.96 ± 0.29	5.51	0.0051	9.80 ± 0.07	2.75	0.001
21	0.23 ± 0.03	6.16 ± 0.31	27.15	0.0001	4.94± 0.426	21.79	0.0008	4.24 ± 0.21	18.71	0.0001

Appendix Chapter 7 Table 16 Proliferative response of sheep PBMC during primary oral infection with 10^5 *T. gondii* oocysts

$S_3$2

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpmx10^3 ± sem	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	0.24 ± 0.03	9.67 ± 0.55	39.17	0.0002	1.56 ± 0.53	6.33	0.044	1.1 ± 0.28	4.44	0.029
-1	0.22 ± 0.06	13.40 ± 0.31	63.37	0.0004	1.53 ± 0.3	6.86	0.01	0.52 ± 0.18	2.33	0.1
0	0.23 ± 0.04	14.14 ± 1	58.21	0	1.31 ± 0.17	5.69	0.022	1.31 ± 0.17	5.69	0.0039
2	0.24 ± 0.015	10.16 ± 0.4	41.02	0.0001	0.3 ± 0.02	1.20	0.072	0.32 ± 0.024	1.29	0.028
5	0.18 ± 0.014	6.50 ± 0.2	35.22	0	0.31 ± 0.039	1.66	0.025	0.29 ± 0.041	1.56	0.045
7	0.10 ± 0.009	8.26 ± 0.61	75.69	0.0005	0.97 ± 0.11	8.88	0.002	0.64 ± 0.11	5.82	0.0096
9	0.079 ± 0.016	12.47 ± 0.33	157.24	0	1.24 ± 0.48	15.60	0.047	0.31 ± 0.03	3.89	0.0031
12	0.077 ± 0.012	5.96 ± 2.6	77.48	0.054	0.21 ± 0.1	2.71	0.13	0.33 ± 0.13	4.31	0.074
14	0.21 ± 0.051	13.00 ± 1.35	59.99	0.0013	1.24 ± 0.51	5.72	0.09	0.33 ± 0.12	1.53	0.21
16	0.26 ± 0.035	10.85 ± 0.4	41.55	0.0001	3.5 ± 0.24	13.41	0.0005	2.55 ± 0.23	9.76	0.0011
19	0.18 ± 0.019	4.32 ± 0.41	23.24	0.001	0.69 ± 0.09	3.70	0.0062	0.73 ± 0.13	3.93	0.013
21	0.27 ± 0.066	9.95 ± 0.68	35.69	0.0004	1.67 ± 0.20	5.98	0.0033	1.49 ± 0.23	5.35	0.0069

Appendix Chapter 7 Table 17 Proliferative response of sheep PBMC during primary oral infection with 10^5 *T. gondii* oocysts

$S_3$3

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpmx10^3 ± sem	cpmx10^3 ±sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	0.55 ± 0.26	9.30 ± 1.43	16.83	0.0044	1.83 ± 0.70	3.32	0.083	3.08 ± 0.19	5.57	0.0004
-1	0.17 ± 0.03	6.10 ± 0.72	35.26	0.0019	0.18 ± 0.03	1.06	0.39	0.18 ± 0.04	1.07	0.40
0	0.15 ± 0.02	11.52 ± 0.43	76.95	0.0001	0.24 ± 0.06	1.61	0.12	0.38 ± 0.05	2.55	0.0087
2	0.35 ± 0.21	10.33 ± 1.17	29.43	0.0017	0.31 ± 0.08	0.89	0.67	0.72 ± 0.41	2.07	0.21
5	0.23 ± 0.037	8.23 ± 0.48	35.47	0.0002	0.52 ± 0.08	2.27	0.017	0.62 ± 0.14	2.68	0.033
7	0.16 ± 0.04	12.63 ± 0.30	74.79	0	1.86 ± 0.47	11.05	0.018	0.86 ± 0.09	5.10	0.0021
9	0.11 ± 0.006	7.44 ± 0.44	65.85	0.0002	0.23 ± 0.05	2.04	0.050	0.15 ± 0.008	1.39	0.0063
12	0.092 ± 0.007	7.44 ± 0.36	80.36	0.0001	0.08 ± 0.03	0.87	0.66	0.07 ± 0.025	0.83	0.71
14	0.077 ± 0.016	16.15 ± 0.62	208.31	0.0001	7.33 ± 1.63	94.55	0.022	2.75 ± 0.91	35.59	0.03
16	0.079 ± 0.008	8.18 ± 0.76	103.14	0.0009	1.50 ± 0.52	18.91	0.036	1.06 ± 0.32	13.44	0.026
19	0.21 ± 0.02	8.06 ± 0.52	37.9	0.0003	5.76 ± 0.78	27.11	0.0028	3.84 ± 0.32	18.06	0.0008
21	0.31 ± 0.04	10.98 ± o.46	34.87	0.0001	5.92 ± 0.23	18.82	0.0001	5.57 ± 0.3	17.68	0.0002

Appendix Chapter 7 Table 18 Proliferative response of sheep PBMC during primary oral infection with 10^5 *T. gondii* oocysts

S_34

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpmx 10^3 ± sem	cpmx 10^3 ± sem	SI	*P* value	cpmx 10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	0.19 ± 0.15	5.72 ± 0.45	28.83	0.0006	0.46 ± 0.14	2.35	0.081	0.41 ± 0.11	2.07	0.081
-1	0.095 ± 0.07	9.78 ± 0.37	102.45	0.0001	0.14 ± 0.008	1.54	0.004	0.12 ± 0.02	1.29	0.17
0	0.17 ± 0.13	13.37 ± 3.89	75.66	0.021	1.21 ± 0.24	6.86	0.024	1.04 ± 0.6	5.92	0.12
2	0.066 ± 0.05	8.96 ± 1.92	134.61	0.0095	0.13 ± 0.02	1.97	0.036	0.21 ± 0.1	3.17	0.12
5	0.16 ± 0.13	2.33 ± 0.17	14.59	0.0004	0.57 ± 0.15	3.59	0.034	0.43 ± 0.03	2.69	0.001
7	0.11 ± 0.08	1.11 ± 0.14	9.85	0.0031	0.15 ± 0.02	1.34	0.095	0.10 ± 0.02	0.96	0.58
9	0.10 ± 0.08	9.26 ± 0.52	90.36	0.0002	0.30 ± 0.03	3.00	0.0027	0.21 ± 0.01	2.07	0.0019
12	0.04 ± 0.03	3.90 ± 1.3	85.27	0.029	0.26 ± 0.04	5.83	0.012	0.29 ± 0.09	6.52	0.05
14	0.04 ± 0.03	11.39 ± 0.48	304.58	0.0001	0.32 ± 0.06	8.52	0.011	0.13 ± 0.03	3.48	0.018
16	0.27 ± 0.2	5.82 ± 0.32	21.18	0.0002	1.19 ± 0.31	4.33	0.031	0.61 ± 0.09	2.22	0.019
19	0.15 ± 0.11	4.04 ± 0.23	26.70	0.0002	0.42 ± 0.09	2.81	0.001	0.29 ± 0.01	1.92	0.001
21	0.22 ± 0.21	6.42 ± 0.67	29.44	0.0013	0.58 ± 0.27	2.66	0.14	0.45 ±0.06	2.07	0.017

Appendix Chapter 7 Table 19 Proliferative response of sheep PBMC during primary oral infection with 10^3 *T. gondii* oocysts

$S_3$5

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpmx10^3 ± sem	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	0.24 ± 0.07	5.59 ± 0.31	23.29	0.0002	0.49 ± 0.1	2.01	0.075	0.50 ± 0.16	2.06	0.21
-1	0.12 ± 0.1	12.07 ± 0.92	100.58	0.0005	0.81 ± 0.4	6.85	0.17	0.52 ± 0.09	4.42	0.023
0	0.14 ± 0.05	8.45 ± 0.54	60.36	0.0003	0.27 ± 0.07	1.94	0.17	0.19 ± 0.005	1.33	0.0034
2	0.09 ± 0.07	3.14 ± 0.18	35.28	0.0002	0.11 ± 0.03	1.27	0.44	0.12 ± 0.03	1.32	0.46
5	0.21 ± 0.16	8.60 ± 0.16	40.95	0	1.19 ± 0.14	5.75	0.0055	0.47 ± 0.06	2.26	0.02
7	0.15 ± 0.12	8.82 ± 0.43	58.80	0.0001	0.82 ± 0.09	5.29	0.005	0.77 ± 0.05	4.97	0.001
9	0.18 ± 0.15	8.74 ± 0.56	48.56	0.0003	4.32 ± 0.32	23.77	0.001	4.14 ± 0.18	22.77	0.0002
12	0.06 ± 0.05	7.21 ± 0.34	120.17	0.0001	0.26 ± 0.01	4.15	0.0026	0.22 ± 0.04	3.63	0.061
14	0.47 ± 0.67	17.38 ± 1.2	36.98	0.0004	6.53 ± 0.21	13.69	0.0001	6.24 ± 0.25	13.09	0.0002
16	0.47 ± 0.39	4.35 ± 0.24	9.26	0.0002	4.34 ± 0.4	9.20	0.0024	3.67 ± 0.27	7.78	0.0012
19	0.33 ± 0.24	2.05 ± 0.24	6.21	0.0028	0.61 ± 0.22	1.86	0.29	0.57 ± 0.13	1.74	0.16
21	0.26 ± 0.2	4.96 ± 0.3	19.08	0.0003	3.13 ± 0.28	12.12	0.0019	2.39 ± 0.28	9.26	0.0049

Appendix Chapter 7 Table 20 Proliferative response of sheep PBMC during primary oral infection with 10^5 *T. gondii* oocysts

$S_3$6

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpmx10^3 ± sem	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	0.25 ± 0.07	8.82 ± 0.64	35.28	0.0004	0.83 ± 0.26	3.32	0.056	0.86 ± 0.23	3.44	0.039
-1	0.29 ± 0.51	18.09 ± 0.5	62.38	0	1.19 ± 0.44	4.10	0.066	1.82 ± 0.73	6.28	0.063
0	0.16 ± 0.05	10.16 ± 0.27	63.50	0	0.54 ± 0.17	3.38	0.056	0.27 ± 0.04	1.69	0.034
2	0.27 ± 0.21	9.60 ± 0.54	35.56	0.0002	0.71 ± 0.2	2.63	0.058	0.64 ± 0.11	2.37	0.025
5	0.58 ± 0.45	11.07 ± 0.5	19.09	0.0001	1.20 ± 0.9	2.07	0.0027	1.14 ± 0.13	1.97	0.012
7	0.19 ± 0.15	7.67 ± 0.2	40.37	0	2.79 ± 0.35	14.68	0.0026	2.24 ± 0.62	11.79	0.023
9	0.12 ± 0.09	11.34 ± 0.45	94.50	0.0001	7.10 ± 0.18	59.17	0	6.93 ± 0.26	57.75	0.0001
12	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
14	0.33 ± 0.25	6.80 ± 0.17	20.61	0	4.99 ± 0.3	15.12	0.0003	4.80 ± 0.22	14.55	0.0001
16	0.31 ± 0.23	12.56 ± 0.4	40.52	0	5.62 ± 0.24	18.13	0.0001	5.44 ± 0.63	17.55	0.002
19	0.30 ± 0.23	8.37 ± 0.3	27.90	0.0001	1.78 ± 0.23	5.93	0.0037	1.26 ± 0.18	4.20	0.0062
21	0.30 ± 0.23	15.92 ± 0.5	53.07	0	5.83 ± 0.24	19.43	0.0001	6.48 ± 0.39	21.60	0.0003

Appendix Chapter 7 Table 21 Proliferative response of cattle PBMC during primary oral infection with 10^5 *T. gondii* oocysts

	$C_4$1									
	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpmx10^3 ± sem	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	0.11 ± 0.02	9.02 ± 1.4	84.87	0.0043	1.95 ± 0.77	18.40	0.048	2.16 ± 1.2	20.30	0.091
-1	0.08 ± 0.03	2.20 ± 0.2	27.84	0.0008	0.15 ± 0.04	1.91	0.08	0.10 ± 0.02	1.34	0.1
0	0.67 ± 0.05	11.35 ± 1.7	16.87	0.0041	8.72 ± 0.8	12.97	0.001	8.24 ± 1	12.25	0.0022
2	0.40 ± 0.06	13.10 ± 1.2	32.47	0.001	7.32 ± 1.4	18.38	0.0078	5.37 ± 1.54	13.49	0.024
5	0.31 ± 0.07	8.26 ± 0.6	26.83	0.0004	0.60 ± 0.14	1.97	0.064	0.45 ± 0.03	1.46	0.012
7	0.07 ± 0.01	12.86 ± 1.1	173.38	0.0007	6.49 ± 0.55	87.51	0.0007	5.50 ± 0.41	74.13	0.0005
9	0.76 ± 0.1	14.07 ± 1.3	18.55	0.001	10.97 ± 1.04	14.46	0.0011	11.03 ± 372.87	14.40	0.0001
12	0.08 ± 0.008	18.71 ± 3.1	239.06	0.0046	3.23 ± 0.58	41.30	0.0061	4.33 ± 0.39	55.35	0.0008
14	0.18 ± 0.07	9.11 ± 1	49.78	0.0014	14.14 ± 2.8	77.27	0.008	14.39 ± 2.61	78.03	0.0062
16	0.22 ± 0.05	6.95 ± 0.67	31.50	0.001	19.07 ± 1.7	86.39	0.0008	16.38 ± 2303.27	74.20	0.003
19	0.50 ± 0.01	2.47 ± 0.38	5.04	0.0066	2.13 ± 0.45	4.34	0.017	1.90 ± 0.24	3.88	0.0047
21	0.79 ± 0.2	7.03 ± 1.34	10.20	0.0089	20.47 ± 1.3	29.71	0.0003	16.50 ± 2.1	23.95	0.0023

Appendix Chapter 7 Table 22 Proliferative response of cattle PBMC during primary oral infection with 10^5 *T. gondii* oocysts

$C_4$2

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpmx10^3 ± sem	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	0.21 ± 0.09	4.42 ± 0.27	21.35	0.0003	8.44 ± 1.53	40.71	0.0064	4.73 ± 0.97	22.84	0.0096
-1	0.05 ± 0.005	8.58 ± 1.1	153.69	0.0021	1.57 ± 0.26	28.15	0.0052	0.83 ± 0.32	14.84	0.049
0	0.14 ± 0.06	13.78 ± 0.65	96.69	0.0001	2.12 ± 0.46	14.87	0.012	3.38 ± 0.36	23.73	0.0015
2	0.05 ± 0.01	9.45 ± 1.31	203.18	0.0028	0.10 ± 0.05	2.17	0.2	0.07 ± 0.02	1.48	0.19
5	0.26 ± 0.02	8.60 ± 0.35	32.85	0.0001	0.95 ± 0.22	3.65	0.027	0.56 ± 0.06	2.14	0.01
7	0.14 ± 0.02	13.16 ± 0.48	93.78	0.0001	5.20 ± 0.03	37.07	0	4.57 ± 0.22	32.58	0.0001
9	3.79 ± 0.62	8.67 ± 1.3	2.29	0.018	10.90 ± 1.4	2.87	0.0073	9.91 ± 0.9	2.61	0.003
12	0.01 ± 0.02	10.54 ± 1.8	753.18	0.005	0.07 ± 0.04	5.56	0.089	0.35 ± 0.14	24.81	0.048
14	0.23 ± 0.06	10.85 ± 1	46.53	0.0009	18.81 ± 2.52	80.68	0.0026	20.99 ± 1.42	90.04	0.0003
16	0.05 ± 0.02	0.98 ± 0.05	19.31	0.0002	0.03 ± 0.07	0.52	0.98	0.03 ± 0.01	0.62	0.9
19	0.34 ± 0.01	1.74 ± 0.17	5.09	0.002	1.62 ± 0.54	4.73	0.05	2.13 ± 0.66	6.24	0.037
21	1.88 ± 0.66	8.34 ± 0.49	4.42	0.0005	18.30 ± 2.2	9.70	0.0024	17.94 ± 2.22	9.51	0.0027

Appendix Chapter 7 Table 23 Proliferative response of cattle PBMC during primary oral infection with 10^5 *T. gondii* oocysts

$C_4$3

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpmx10^3 ± sem	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	1.63 ± 0.17	6.01 ± 0.6	3.69	0.0032	1.30 ± 0.6	0.80	0.68	0.73 ± 0.24	0.45	0.98
-1	0.44 ± 0.08	9.02 ± 1	20.61	0.0015	1.41 ± 0.31	3.21	0.026	2.25 ± 0.56	5.15	0.024
0	2.03 ± 0.6	11.36 ± 0.5	5.59	0.0003	5.01 ± 1.85	2.47	0.1	6.26 ± 1.32	3.08	0.025
2	0.08 ± 0.01	13.44 ± 0.8	173.54	0.0002	0.14 ± 0.05	1.81	0.15	0.097 ± 0.01	1.26	0.088
5	0.24 ± 0.009	7.14 ± 0.3	29.18	0.0001	0.82 ± 0.08	3.35	0.0024	0.69 ± 0.13	2.82	0.021
7	0.23 ± 0.03	10.02 ± 0.6	44.50	0.0003	3.85 ± 1.7	17.11	0.064	1.93 ± 0.1	8.58	0.0002
9	1.13 ± 0.2	5.16 ± 0.5	4.55	0.0005	3.85 ± 0.4	3.40	0.0033	4.60 ± 0.76	4.06	0.0098
12	0.05 ± 0.004	12.65 ± 1	243.23	0.0004	1.92 ± 0.73	36.82	0.042	3.82 ± 1.3	73.47	0.03
14	0.56 ± 0.17	11.98 ± 0.56	21.29	0.0001	18.88 ± 1.02	33.55	0.0002	18.91 ± 0.95	33.61	0.0002
16	0.02 ± 0.0008	2.63 ± 0.11	160.15	0.0001	0.05± 0.03	3.36	0.14	0.02 ± 0.002	0.99	0.51
19	1.24 ± 0.23	2.82 ± 0.09	2.28	0.0002	3.02 ± 0.5	2.44	0.018	3.17 ± 0.3	2.56	0.0048
21	0.29 ± 0.08	11.14 ± 1	38.14	0.0008	10.58 ± 1.6	36.23	0.004	9.64 ± 0.9	33.01	0.0011

Appendix Chapter 7 Table 24 Proliferative response of cattle PBMC during primary oral infection with 10^5 *T. gondii* oocysts

$C_4$4

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpmx10^3 ± sem	cpmx10^3 ±sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	1.37 ± 0.16	4.07 ± 0.39	2.98	0.003	5.06 ± 0.61	3.70	0.0045	3.26 ± 0.67	2.39	0.033
-1	0.70 ± 0.04	5.31 ± 0.59	7.58	0.0021	5.03 ± 0.41	7.19	0.0009	3.87 ± 0.38	5.53	0.0017
0	0.96 ± 0.09	7.85 ± 0.5	8.16	0.0004	8.15 ± 0.58	8.48	0.0006	7.71 ± 0.9	8.02	0.0022
2	0.46 ± 0.12	6.71 ± 0.62	14.58	0.001	2.03 ± 0.5	4.42	0.026	1.43 ± 0.34	3.12	0.032
5	0.41 ± 0.025	4.70 ± 0.45	11.41	0.0012	3.71 ± 0.32	9.01	0.001	3.86 ± 0.23	9.37	0.0003
7	0.35 ± 0.07	9.00 ± 0.57	25.42	0.0003	14.40 ± 0.38	40.64	0	11.74 ± 0.4	33.15	0
9	1.81 ± 0.37	5.92 ± 0.45	3.28	0.0014	16.02 ± 0.68	8.87	0.0001	13.94 ± 0.54	7.72	0.0001
12	0.41 ± 0.04	7.71 ± 0.5	18.86	0.0003	14.96 ± 1.37	36.59	0.0009	15.13 ± 1.21	36.99	0.0006
14	3.92 ± 3.18	7.83 ± 0.68	2.00	0.0053	5.40 ± 1.94	1.38	0.25	7.75 ± 3.9	1.98	0.2
16	2.28 ± 0.46	15.34 ± 0.56	6.73	0.0001	21.51 ± 0.85	9.44	0.0001	21.64 ± 0.7	9.49	0.13
19	0.32 ± 0.06	1.81 ± 0.21	5.71	0.003	0.31 ± 0.06	0.97	0.55	0.53 ± 0.05	1.66	0.015
21	0.65 ± 0.12	12.88 ± 0.64	19.82	0.0002	7.70 ± 2.08	11.98	0.021	8.44 ± 0.79	12.98	0.0011

Appendix Chapter 7 Table 25 Proliferative response of cattle PBMC during primary oral infection with 10^5 *T. gondii* oocysts

$C_4$5

	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5μg/ml			12.5μg/ml			6.25μg/ml		
Days of infection	cpmx10^3 ± sem	cpmx10^3 ±sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	0.11 ± 0.04	8.69 ± 0.92	76.21	0.0013	6.95 ± 1.8	60.89	0.016	5.52 ± 1.8	48.40	0.027
-1	0.04 ± 0.02	0.78 ± 0.2	20.96	0.013	0.04 ± 0.02	1.14	0.39	0.04 ± 0.005	1.10	0.25
0	1.48 ± 1.12	14.74 ± 1.2	9.93	0.0008	8.03 ± 1.36	5.42	0.0086	5.98 ± 0.9	4.03	0.0076
2	0.08 ± 0.02	7.91 ± 0.22	93.55	0	1.73 ± 0.51	20.47	0.024	1.83 ± 0.9	21.64	0.068
5	0.18 ± 0.01	5.71 ± 0.3	31.67	0.0002	1.42 ± 0.18	7.88	0.0032	0.55 ± 0.09	3.07	0.014
7	0.13 ± 0.03	5.35 ± 0.63	40.56	0.0018	2.63 ± 0.11	19.96	0.0001	2.34 ± 0.1	17.75	0.0001
9	0.18 ± 0.011	9.02 ± 0.24	49.75	0	8.63 ± 0.35	47.61	0.0001	10.66 ± 0.5	58.83	0.0001
12	0.09 ± 0.01	10.13 ± 0.97	108.6	0.0009	8.35 ± 0.85	89.56	0.0012	5.69 ± 1.1	61.04	0.0069
14	0.39 ± 0.9	4.53 ± 0.15	11.7	0.0001	8.62 ± 0.26	22.29	0	7.06 ± 0.64	18.25	0.0009
16	0.38 ± 0.04	11.12 ± 0.51	28.95	0.0001	13.49 ± 0.4	35.10	0	12.1 ± 1.7	31.44	0.0033
19	0.08 ± 0.01	4.50 ± 0.8	59.05	0.0051	0.89 ± 0.2	11.74	0.014	1.27 ± 0.5	16.68	0.046
21	0.38 ± 0.1	13.86 ± 0.66	36.28	0.0001	18.33 ± 1.62	47.97	0.0008	14.75 ± 2.1	38.63	0.0032

Appendix Chapter 7 Table 26 Proliferative response of cattle PBMC during primary oral infection with 10^5 *T. gondii* oocysts

	$C_4$6									
	Controls				water soluble fraction of S48 *T. gondii* antigen					
	Medium	Con-A 5mg/ml			12.5mg/ml			6.25mg/ml		
Days of infection	cpmx10^3 ± sem	cpmx10^3 ±sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value	cpmx10^3 ± sem	SI	*P* value
-2	0.08 ± 0.008	5.39 ± 0.95	66.75	0.0056	0.44 ± 0.1	5.50	0.019	0.29 ± 0.1	3.63	0.068
-1	1.23 ± 0.42	14.7 ± 2.7	11.99	0.0076	4.56 ± 0.48	3.72	0.003	4.83 ± 1.2	3.94	0.028
0	0.51 ± 0.07	16.96 ± 0.8	33.58	0.0001	3.18 ± 0.7	6.30	0.033	2.08 ± 0.45	4.13	0.02
2	0.18 ± 0.013	8.64 ± 1	46.54	0.0017	1.16 ± 0.2	6.27	0.0098	0.74 ± 0.14	3.96	0.016
5	0.16 ± 0.02	4.19 ± 0.4	26.08	0.0008	0.28 ± 0.05	1.74	0.046	0.29 ± 0.05	1.81	0.043
7	0.13 ± 0.009	6.13 ± 0.5	48.04	0.0006	0.56 ± 0.06	4.37	0.0024	0.52 ± 0.09	4.11	0.0097
9	0.99 ± 0.2	5.15 ± 0.9	5.23	0.0067	6.70 ± 0.9	6.80	0.0037	5.47 ± 0.4	5.56	0.0006
12	0.28 ± 0.03	7.97 ± 1.52	28.02	0.0074	2.44 ± 0.25	8.60	0.0064	3.15 ± 0.84	11.06	0.021
14	0.17 ± 0.1	11.25 ± 0.6	67.28	0.0002	8.74 ± 1.3	52.24	0.0034	8.69 ± 0.8	51.96	0.0008
16	0.20 ± 0.04	10.19 ± 0.9	34.14	0.0007	6.77 ± 0.14	22.67	0.0003	4.46 ± 0.4	14.95	0.0009
19	0.37 ± 0.08	3.26 ± 0.26	8.79	0.0007	1.39 ± 0.1	3.75	0.0049	0.97 ± 0.2	2.60	0.025
21	0.27 ± 0.05	8.85 ± 0.2	32.63	0	9.92 ± 1.8	36.57	0.016	2.44 ± 0.6	9.00	0.022

Appendix

Publications and Proceedings of meetings

Publications

Innes, E. A. and **Esteban, I**. (1997) Diagnosis of toxoplasmosis. Aula Veterinaria. Tratado de Patología y Producción Ovina. (*In press*).

Esteban-Redondo, I. and Innes, E.A. (1997) *Toxoplasma gondii* infection in sheep and cattle. *Comparative Immunology, Microbiology and Infectious Diseases* (*In press*).

Proceedings of meetings

Esteban, I. and Innes, E.A. (1993). A comparison of serological responses of cattle and sheep to *Toxoplasma gondii*. Third International Sheep Veterinary Conference. Edinburgh (UK).

Esteban, I, Buxton, D. and Innes, E.A. (1995). Comparison of immune responses and pathogenesis of *Toxoplasma gondii* infection in cattle and sheep. Comparative and Veterinary Immunology Group. Workshop on immunology of zoonotic infections. British Society of Immunology. Birmingham (UK). **Invited speaker.**

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TOXOPLASMA GONDII INFECTION IN SHEEP AND CATTLE

IRMA ESTEBAN-REDONDO* and ELISABETH A. INNES
Moredun Research Institute, 408 Gilmerton Road, Edinburgh, EH17 7JH, U.K.

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Abstract—*Toxoplasma gondii* is a protozoan parasite that can infect all warm-blooded animals. Sheep and cattle show different susceptibilities to *T. gondii* infection. Primary infection in pregnant sheep can result in abortion or the birth of weak lambs but they are then protected against further challenge by the development of an effective immunity. Cattle on the other hand can be readily infected, but abortion or perinatal mortality have not been recorded. The evidence suggests that cattle develop a more effective immune response to *T. gondii* infection than sheep. Potential mechanisms to explain these differences are discussed in this paper. Published by Elsevier Science Ltd

Key words: Toxoplasma gondii, sheep, cattle

Résumé—Le *Toxoplasma gondii* est un protozoo parasite qui peut infecter tous les animaux de sang chaude. Le mouton et les bovines sont differentment sousceptibles à l'infection pour *T. gondii*. L'infection primaire chez le mouton pendant la pèriode de gestation peut provoquer l'abortement ou la naissance du animaux faibles mais ils sont alors protegés contre une infection posterieur par le dévélopement d'une immunité effective. Bovines, par contre, peuvent être vraiment infectés mais ni l'abortement ni la mortalité périnatal ont été décrités. L'evidence sugére que les bovines dévelopent une résponse immune plus éffective contre le *T. gondii* que le mouton. Les posibles mecanismes pour expliquer ces difference sont discutés dans ce article. Published by Elsevier Science Ltd

Mots-clefs: Toxoplasma gondii, mouton, bovines

INTRODUCTION

Toxoplasma gondii, a protozoan parasite, was described for the first time in 1908. While the sexual life cycle of the parasite is confined to cats (the definitive host), the asexual cycle can take place in all warm-blooded animals but causes disease only in certain species [1]. Cattle, like deer and horses, are susceptible to infection but resistant to disease induced by *T. gondii*, whereas the parasite can cause a lethal infection in the developing fetus of sheep. *Toxoplasma* also has been recognised as one of the most important opportunistic pathogens in AIDS patients, in whom it can cause a severe, non-suppurative, necrotising encephalitis.

Abortion and neonatal disease in sheep and humans result from primary infection occurring during pregnancy [2], but immunity developed after such an episode will protect the mother against subsequent challenge [3, 4]. Despite this immune response, the parasite commonly persists in the ovine and human maternal tissues for life.

*Author for correspondence.

The ingestion of meat containing viable *T. gondii* tissue cysts is an important source of human infection [1]. While the parasite has been isolated frequently from sheep meat, it is not so readily isolated from cattle meat [5]. The reasons for this difference are unclear and the objective of the work reported in this communication was to compare the course of *T. gondii* infection in sheep and cattle.

Toxoplasma gondii: *life cycle*

Toxoplasma gondii is an obligate, intracellular, protozoan parasite, with a sexual life cycle confined to the enteroepithelium of Felidae, resulting in the production of oocysts (Fig. 1). The asexual cycle, with little host specificity, comprises two developmental stages, rapidly dividing tachyzoites and slowly multiplying bradyzoites [1]. Tachyzoites actively penetrate host cells and are surrounded by a parasitophorous vacuole in which they multiply by endodyogeny before breaking out to infect other cells. The process continues until the host develops immunity to the parasite [6]. Normally, a persistent infection is established, extracellular parasites are eliminated, intracellular multiplication slows down and the parasite remains in the host as bradyzoites contained within tissue cysts. These are most frequently detected in brain, heart and skeletal muscle. The host remains persistently infected but immune to further challenge by the parasite [1].

The sexual cycle is initiated when a cat ingests *Toxoplasma* tissue cysts for the first time to release bradyzoites in the small intestine. These penetrate the gut epithelial cells and undergo merogony before gametogeny commences. The development and subsequent fusing of microgametes with macrogametes results in the formation of oocysts, which are usually excreted in the faeces between the fourth and tenth days. After sporulation outside the host, oocysts become infectious and may remain so for many months in the environment [1].

Clinical toxoplasmosis

Sheep. Toxoplasmosis has been recognised as a major cause of abortion in sheep since 1951, when *T. gondii*-like structures were described in placentas and aborted fetuses in New Zealand [7]. Abortions may occur in ewes of all ages when infection is acquired for the first time during pregnancy. Subsequently, ewes remain infected for life but will not abort from this cause again [4].

The modes of transmission of *T. gondii* to herbivores are either transplacental or by ingestion of fodder or concentrate rations contaminated with oocysts. The oocysts, after

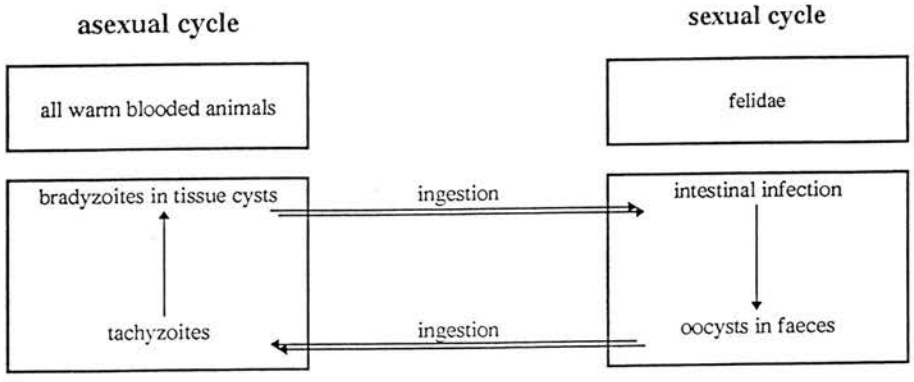

Fig. 1. Life cycle of *Toxoplasma gondii*.

excysting in the small intestine, release sporozoites, which reach the mesenteric lymph node within 4 days of infection [8]. Experiments suggest that the tachyzoites become widely disseminated during the parasitaemia that occurs between the fifth and the twelfth days of infection [9, 10]. This is followed by the onset of a protective immune response, although the organism then persists as bradyzoites within tissue cysts in a variety of organs [11]. Infection is generally unapparent, although fever may be detected in the initial days. However, if established for the first time during pregnancy, a progressive infection may establish in the gravid uterus [11]. In these circumstances, tachyzoites invade the caruncular septa, the maternal tissue of the placentome, and then invade the placental villi and the fetus [12]. The ability of the fetal immune system to respond to *T. gondii* develops progressively after 70 days of gestation, so that infection before this age results in rapid fetal death with resorption, mummification, maceration or abortion of the fetus. Infection later in pregnancy may be less damaging and result in stillborn or weak lambs, or even clinically normal lambs that are infected and immune to natural challenge [12]. Stillborn or weak lambs usually have brain damage such as focal leukomalacia and a characteristic non-suppurative meningo-encephalitis [13]. Lambs that survive the first few days of life generally grow normally to adulthood without neurological defect [11].

Cattle. In 1953, what was thought to be clinical toxoplasmosis in cattle was reported for the first time in the United States. The clinical features and demonstration of *T. gondii*-like structures were reported in four separate herds, in which some of the adult cattle died but greater losses occurred in young animals. Parasites were demonstrated in tissue sections from calves and cows. The surviving animals had a positive reaction to the intradermal toxoplasmin test and respiratory and central nervous system symptoms were described [14]. These findings have been challenged since further examinations of the tissues failed to reveal *T. gondii* or related protozoan parasites [15]. Other studies of natural and experimental toxoplasmosis in cattle carried out since 1953 have shown that toxoplasmosis does not appear to cause abortion or neonatal mortality in cattle under natural conditions [15, 17]. However, in several cases the parasite has been isolated in mice inoculated with pooled homogenates of tissues from cattle with suspected infection, and from colostrum or unpasteurised milk, indicating that beef and milk cannot be ruled out as potential reservoirs of infection in the epidemiology of the disease [15]. Pregnant cattle inoculated with *T. gondii* oocysts or tissue cysts developed transient fever and anorexia and gave birth to healthy calves. In this case the parasite was not isolated from the tissues of the newborn calves or placentas [16]. However, after experimental infection of four calves with *T. gondii* tachyzoites, two had lesions in the central nervous system when examined within ten days after infection and the parasite was isolated from several of their tissues [17]. Other groups have studied toxoplasmosis in cattle by the administration of tachyzoites, tissue cysts or oocysts of the parasite by several routes (orally, parenterally, intra-amniotically or intra-ruminally). In general, animals developed a febrile response and loss of appetite. Some had diarrhoea and respiratory distress, but recovered within 3 weeks. In only four of eight calves orally inoculated with oocysts was *T. gondii* subsequently found in the mesenteric lymph nodes 3 and 6 days later [15].

Humoral response

In sheep, the serological diagnosis of the infection is conventionally made by an immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA). In this species, following both experimental and natural infection with *T. gondii*,

specific antibody titres rise 2–3 weeks after infection and remain elevated for several years [18]. IgM is more readily detected during the first month, with IgG becoming the predominant immunoglobulin subclass during the second month of infection; reinfection does not elicit an increase in antibody titre [4, 18]. Seroprevalence in sheep increases with age, reflecting increased exposure to the parasite over time [1].

In cattle, serum antibodies to *T. gondii* have been found world-wide, although the actual prevalence rates are difficult to ascertain [1]. The Sabin–Feldman dye test (DT) is probably not suitable for use in cattle as it may give a false-positive reaction owing to a naturally occurring globulin, thought to be IgM. This globulin can be partially inactivated by heating the sera to 60°C for 60 min [19]. Furthermore, in experimentally infected cattle, DT titres increase briefly during the first month after inoculation before returning to baseline levels. By comparison, the modified agglutination test (MAT) titres for the same samples increased dramatically during the first 4 weeks and persisted for several months. In this context it may be relevant that live tachyzoites are used in the DT, whereas the MAT relies on dead organisms [19].

In cattle there is also an age effect in antibody development, with calves producing higher and longer lasting antibody titres than adult cattle, following experimental challenge with the same dose of *T. gondii* [15, 19]. Under natural conditions it has been shown that sheep grazing on a pasture alongside cattle developed significant antibody titres to *T. gondii*, as measured by IFAT, while none of the calves became seropositive [20]. In addition, a study of seroprevalence in cattle in Montana, U.S.A., showed that most naturally infected animals had only low titres of antibody to *T. gondii* [1]. Thus, sheep and cattle appear to differ in their susceptibility and humoral responses to the parasite.

It is concluded that the differences in humoral responses to *T. gondii* between sheep and cattle as measured by IFAT are real, as this test has been shown to be appropriate for use in both species [21].

Cell-mediated immunity

As *T. gondii* is an intracellular parasite, cell-mediated immunity (CMI) would be expected to play a major role in host defence. In sheep, the incomplete S48 strain of *T. gondii*, which lacks the ability to produce tissue cysts (and therefore to establish persistent infection), has been used in experimental infections [22]. Following subcutaneous inoculation of naive sheep with S48 tachyzoites, phenotypic analysis of lymphoblasts in the efferent lymph showed that the cells responding to infection were mainly T-cells. Initially those with a CD4 + phenotype were more numerous than CD8 + cells but around the ninth or tenth day after infection the latter became predominant. The disappearance of the parasite from the lymph also occurred at this time [23]. Interferon gamma (IFN-γ) was detected in cell-free lymph supernatant from the third to the tenth day after primary infection but appeared in lymph as early as 24 h after secondary challenge [24]. Both CD4 + and CD8 + T-cell subsets have the potential to produce IFN-γ after appropriate stimulation [25, 26], but the source of IFN-γ in the sheep following *T. gondii* infection is not yet known. These observations suggest that a T-cell response combined with IFN-γ production plays a major role in immunity to *T. gondii* infection in sheep. These *in vivo* observations are further supported by *in vitro* studies, which have shown that recombinant ovine IFN-γ will inhibit the growth of *T. gondii* in ovine cells [27].

Studies of CMI in cattle in response to *T. gondii* infection have not been published, however, in view of the perceived importance of CD4 + and CD8 + cells and IFN-γ in

ovine immunity to the parasite, it would be of value to make comparative studies in cattle and combine those results with investigations of the humoral responses in both species.

Tissue cyst development

Toxoplasma gondii tissue cysts have been isolated frequently from the tissues of naturally and experimentally infected sheep [1]. Viable parasites have been detected by inoculation of mice or cats with soft tissues such as brain, skeletal muscle, small intestine, liver and diaphragm. However, the brain is the most heavily infected tissue in aborted fetuses or live lambs born to infected ewes and should be the organ of choice for isolating *T. gondii* for diagnostic purposes [28].

The parasite has been isolated only rarely from naturally infected bovine tissues although there have been occasional reports from Japan, Czechoslovakia, Italy and Argentina of successful isolations [15]. The parasite has been recovered from the intestinal wall of a naturally infected adult cow [5] and a recent report has stated that *T. gondii* can remain viable in tissues of experimentally infected cattle for more than 3 years [29]. The most sensitive method of isolating *T. gondii* from infected cattle is by feeding cats with tissues and subsequently demonstrating oocysts in the faeces of the latter. The tissues to be examined in this way should include the heart, tongue, intestines, liver [29] and brain (unpublished data).

In an attempt to study the distribution of *T. gondii* cysts in cattle tissues, Dubey dosed calves orally with 100 000 oocysts of the GT-1 strain of *T. gondii* [30]. Samples of small intestine, mesenteric lymph nodes, lung and liver of infected calves were sampled after 3, 6, 8, 11 and 14 days and fed to mice to determine the prevalence of the parasite in the tissues. It was found that *T. gondii* was present in the small intestine and mesenteric lymph nodes on all the days tested. It was not detected in liver on day 8 and lung on days 8 and 11 but was recovered from both tissues at all the other times points tested. The number of recovered live organisms diminished rapidly within the first week after inoculation and the parasite was recovered more frequently from liver than from muscle, and not at all from blood or retina.

CONCLUSION

Toxoplasma gondii infection affects all warm-blooded animals with a wide species variation in the disease it causes. The outcome of a primary infection in pregnant sheep may result in abortion or neonatal infection, while in cattle the parasite is eliminated quickly from the tissues and clinical abortion has not been reported.

The factors that confer this natural resistance or susceptibility to toxoplasmosis are not yet known. Comparative studies on *T. gondii* infection in sheep and cattle may offer a valuable insight into the development of protective immunity to the parasite.

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